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Xylose Fermentation to Ethanol: A Review

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Summary

The past several years have seen tremendous progress in the understanding of xylose metabolism and in the identification, characterization, and development of strains with improved xylose fermentation characteristics. A survey of the numerous microorganisms capable of directly fermenting xylose to ethanol indicates that wild-type yeast and recombinant bacteria offer the best overall performance in terms of high yield, final ethanol concentration, and volumetric productivity. The best performing bacteria, yeast, and fungi can achieve yields greater than 0.4 g/g and final ethanol concentrations approaching 5% (w/v). Productivities remain low for most yeast and particularly for fungi, but volumetric productivities exceeding 1.0 g/L-h have been reported for xylose-fermenting bacteria. In terms of wild-type microorganisms, strains of the yeast *Pichia stipitis* show the most promise in the short term for direct high-yield fermentation of xylose without byproduct formation. Of the recombinant xylose-fermenting microorganisms developed, recombinant *E. coli* ATTC 11303 (pLOI297) exhibits the most favorable performance characteristics reported to date. Several fungi and thermophilic bacteria offer long-term promise and should continue to be examined.

There are disadvantages to the use of either *P. stipitis* or recombinant *E. coli*. The primary disadvantage with *P. stipitis* is that very low levels of aeration are necessary to achieve optimal performance. Recombinant *E. coli* ATTC 11303 (pLOI297) suffers from the fact that its genetic stability is unproven in prolonged culture, there is a higher probability of contamination because it must be cultivated at nearly neutral pH, and regulatory obstacles may hinder its large-scale use.

Developments in xylose conversion are progressing rapidly, although many questions remain regarding key aspects of xylose metabolism in bacteria, yeast, and fungi. A large body of literature already exists concerning the performance of xylose-fermenting yeast. Data on the performance of recombinant bacteria are accumulating quickly.

The construction of recombinant bacterial and yeast strains that exhibit improved xylose fermentation performance characteristics represents the most significant recent development in xylose conversion research. Genetic engineering work aimed at constructing a recombinant *Saccharomyces cerevisiae* strain capable of high-yield xylose fermentation is nearing fruition. Progress is being made on developing improved recombinant bacterial strains. Given the rapid pace of advances being made in metabolic engineering of improved xylose-fermenting organisms, the development of recombinant organisms capable of much improved performance seems imminent.

Substantial progress is being made in achieving efficient high-yield xylose fermentation and the successful development of large-scale processes appears likely within the decade.

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Introduction

Because the pentose sugar D-xylose comprises about one-third of the total carbohydrate sugars in lignocellulosic biomass, favorable economics for large-scale production of ethanol from lignocellulosic materials require efficient conversion of xylose. The U.S. Department of Energy (DOE)/National Renewable Energy Laboratory (NREL) Ethanol from Biomass Program is evaluating a variety of fermentation-based bioconversion processes for converting xylose (and other pentose sugars) to ethanol at an industrial scale. An economic analysis of xylose fermentation conducted by Hinman et al. (1989) concludes that for fixed substrate costs, ethanol yield and final concentration most influence ethanol production costs, with ethanol production rate of secondary importance. Thus, efforts are focused on determining the microorganisms that are most capable of carrying out high-yield xylose fermentation to high ethanol concentration.

The past several years have seen tremendous progress in the understanding of xylose metabolism and in the identification, characterization, and development of strains with improved xylose fermentation characteristics. This report reviews the current state of xylose fermentation technology, focusing on recent improvements in the direct fermentation of xylose (developments involving xylanases and simultaneous isomerization and fermentation are not discussed). Anticipated advantages and disadvantages of processes currently being considered for large-scale development are discussed and recommendations are made concerning directions for future xylose conversion research.

Background and Historical Overview

Microorganisms

Microorganisms that rapidly ferment xylose to ethanol at high yields are essential to the development of cost-effective, large-scale xylose to ethanol processes. The ability of certain fungi and bacteria to ferment xylose to ethanol has been recognized for many years (Chaing and Knight 1960; Horecker 1962). Interest in commercially exploiting this technology grew rapidly during the 1980s and many laboratories undertook isolation and screening programs to identify xylose-fermenting microbes (Maleszka and Schneider 1982; Suihko and Dražić 1983; Toivola et al. 1984; du Preez and Prior 1985; Morikawa et al. 1985; Nigam et al. 1985). As documented in recent reviews of xylose fermentation, numerous microorganisms are now recognized to be capable of carrying out direct fermentation of xylose to ethanol (Jeffries 1985a; Magee and Kosaric 1985; Manderson 1985; Skoog and Hahn-Hägerdal 1988; Schneider 1989; Jeffries 1990; Hahn-Hägerdal et al. 1991). Table 1 lists microbiological genera that contain species capable of direct xylose fermentation. Also listed are several recombinant bacterial and yeast genera incapable of direct high-yield xylose fermentation in their native genotype that are being used as hosts in genetic engineering research aimed at developing improved xylose-fermenting microorganisms (see discussion in the section on genetic engineering advances below).

Yeast genera with the capacity for direct fermentation of xylose include *Brettanomyces, Candida, Clavispora, Kluyveromyces, Pachysolen, Pichia*, and *Schizosaccharomyces*. There is a general consensus in the literature that *Candida shehatae, Pachysolen tannophilus*, and *Pichia stipitis* are the three native (wild-type) yeast species best able to carry out high-yield xylose fermentation. Although opinions differ regarding which of these species is best suited for use in a commercial process, recent yeast-based xylose conversion research has largely focused on characterizing the xylose fermentation performance of these three yeasts.

Table 1. Genera Capable of Direct Fermentation of Xylose to Ethanol

YEAST	WILD-TYPE GENERA	
		Brettanomyces
		Candida
		Clavispora
		Kluyveromyces
		Pachysolen
		Pichia
		Schizosaccharomyces
	RECOMBINANT GENERA	
		Saccharomyces
		Schizosaccharomyces
FUNGI	WILD-TYPE GENERA	
		Fusarium
		Monilia
		Mucor
		Neurospora
		Paecilomyces
		Polyporus
		Rhizopus
BACTERIA	WILD-TYPE GENERA	
		Aerobacter
		Aeromonas
		Bacillus
		Bacteroides
		Clostridium
		Erwinia
		Klebsiella
		Thermoanaerobacter
	RECOMBINANT GENERA	
		Erwinia
		Escherichia
		Klebsiella

A variety of wild-type fungal and bacterial genera can carry out direct fermentation of xylose. Fungal genera, which often exhibit high yields but suffer from low rates, include *Fusarium, Monilia, Mucor, Neurospora, Paecilomyces, Polyporus*, and *Rhizopus*. Bacterial species are found in both mesophilic and thermophilic genera. Mesophilic genera include *Aerobacter, Aeromonas, Bacillus, Bacteroides, Erwinia*, and *Klebsiella*. The two thermophilic genera identified to date are *Clostridium* and *Thermoanaerobacter*. Bacteria are generally recognized to provide high rates at the expense of low ethanol yields; wild-type bacteria carry out mixed product fermentations that are characterized by low ethanol yield. Research into using wild-type bacteria and fungi to convert xylose waned during the 1980s due in part to the widely recognized disadvantages of low rate and/or poor yield of these types of microorganisms coupled with the fact that highly productive xylose-fermenting yeasts had been identified. The bulk of recent research on bacteria has been directed at constructing recombinant strains capable of direct xylose fermentation, as elaborated on below. Recent fermentation-based research on wild-type bacteria has largely focused on examining thermophilic species for use in direct microbial conversion (DMC) processes. Although relatively little research was performed in the 1980s on direct xylose fermentation by fungi, research on fungi has been renewed in the 1990s.

Metabolism

Extensive reviews of xylose metabolism are available (e.g., Chaing and Knight 1960; Horecker 1962; Jeffries 1983; McCracken and Gong 1983; Magee and Kosaric 1985; Manderson 1985; Jeffries 1990). The major metabolic pathways for xylose fermentation are similar in bacteria, yeast, and fungi with the notable exceptions of significant differences in transport, regulation, cofactor requirements, and the products of pyruvate fermentation. After transport into the cell and conversion to xylulose-5-phosphate, ethanol production from xylose is believed to proceed by way of the pentose phosphate (PP) and Embden-Meyerhof-Parnas (EMP) pathways, as depicted in Figure 1. Xylose must be converted to xylulose and then phosphorylated to xylulose-5-phosphate before entering the PP cycle. Within the PP cycle, xylulose-5-phosphate is metabolized to glyceraldehyde-3-phosphate and fructose-6-phosphate, then these compounds are converted to pyruvate in the EMP pathway. The pyruvate is finally converted to ethanol. It is relevant to note that the method by which pyruvate is converted to acetaldehyde is different in yeast than in most bacteria and fungi, although all microorganisms employ alcohol dehydrogenase to convert acetaldehyde to ethanol. In the overall fermentative reaction, 3 moles of xylose are required to produce 5 moles of ethanol. The stoichiometry, neglecting the NAD(P)H balance, is shown in Equation (1).

$$3XYLOSE + 5ADP + 5P_i \rightarrow 5ETHANOL + 5CO_2 + 5ATP + 5H_2O \quad (1)$$

The theoretical ethanol based on this stoichiometry is 0.51 g ethanol/g xylose or 1.67 mol ethanol/mol xylose.

Two alternative pathways for xylose metabolism, known as the phosphoketolase and oxidative bypasses, also exist in yeast and fungi (McCracken and Gong 1983; Jeffries 1990). These pathways may also be present in many bacteria (Gottschalk 1986). The phosphoketolase bypass converts xylulose-5-phosphate to glyceraldehyde-3-phosphate and acetylphosphate. Glyceraldehyde-3-phosphate enters glycolysis, whereas acetylphosphate is converted to acetate with the concomitant formation of ATP. Phosphoketolase is known to be present in *Pa. tannophilus*, and it has been speculated that this pathway is important for ATP generation, particularly under anaerobic conditions (Evans and Ratledge 1984; Jeffries 1990). Phosphoketolase is a key enzyme in the heterofermentive pathway used by lactic acid bacteria. Some homofermentative lactobacilli are also capable of fermenting pentoses by this pathway (Gottschalk 1986). The oxidative bypass oxidizes fructose-6-phosphate to ribulose-5-phosphate (ribulose-5-Ⓟ) and carbon dioxide through the intermediate 6-phospho-gluconate (Bruinenberg et al. 1983; Evans and Ratledge 1984;

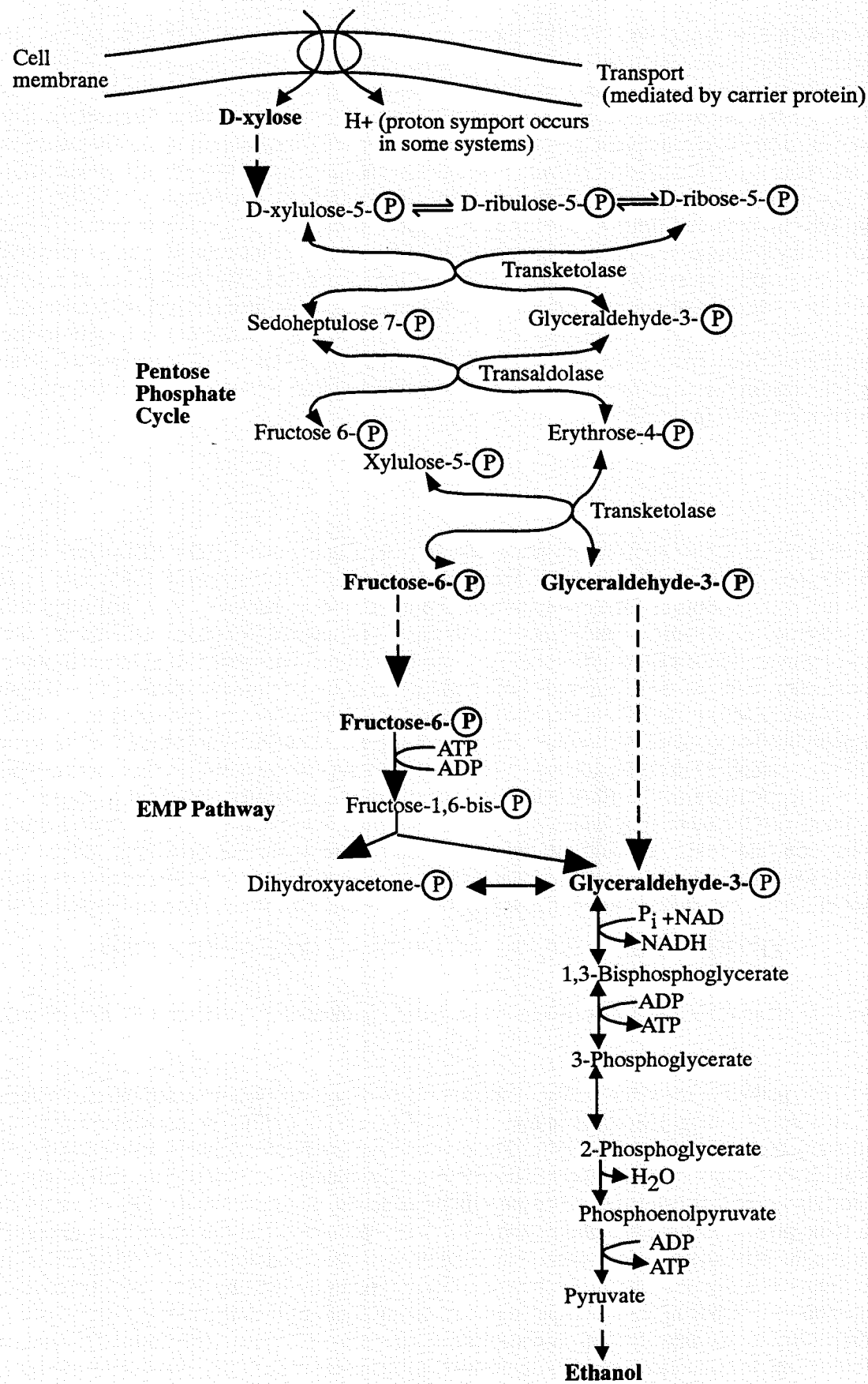

Figure 1. Common elements of D-xylose metabolism to ethanol

Jeffries 1990). Two moles of NADP are reduced to NADPH for each mole of fructose-6-Ⓟ oxidized to ribulose-5-Ⓟ. Obviously, both pathways result in reduced ethanol yield and need to be avoided to achieve high-yield ethanol production.

Transport

Sugar transport across the cell membrane and the regulation of the various sugar transport systems play a key role in determining how productively microorganisms ferment xylose. The general importance of sugar transport is evidenced by the fact that transport and phosphorylation of glucose have previously been proposed as the rate-limiting steps in glycolysis (Jeffries 1983; Bailey and Shanks 1991). It is, therefore, likely that sugar transport may also limit the rate of ethanol production from xylose in xylose-fermenting yeasts. Kilian and van Uden (1988) hypothesized that transport limited the rate of ethanol production in aerobically grown *P. stipitis*. Ligthelm et al. (1988a) concluded that xylose transport is rate limiting during aerobic growth of *P. stipitis*, but that xylitol dehydrogenase activity is rate limiting under anaerobic conditions. Studies by Alexander et al. (1988a) suggest that xylose transport limits the rate of xylose metabolism in *C. shehatae* under fully aerobic and oxygen-limited conditions.

The characteristics of **D**-xylose transport systems in different microorganisms are reviewed by Jeffries (1983) and Webb and Lee (1990). Microorganisms can utilize facilitated diffusion and/or active (energy-requiring) mechanisms for sugar uptake. Active transport systems, in contrast to facilitated diffusion systems, require metabolic energy and can uptake sugars against a concentration gradient. Active transport systems are categorized by the type of metabolic energy they use: chemosmotic, direct energization, or group translocation. Because sugar uptake by both facilitated-diffusion- and active-transport-based systems is mediated by carrier proteins, such systems are characterized by high substrate specificity and a maximum rate determined by the expression and activity level of the carrier protein and/or other enzymes involved in the transport system (and in the case of facilitated diffusion by the transmembrane concentration gradient). Depending on the degree of specificity of the carrier protein, uptake systems may be subject to competitive inhibition by other sugars or sugar analogues. Many xylose uptake systems are based on chemosmotic proton symport and are thus strongly pH dependent.

Bacteria commonly employ active (energy-requiring) mechanisms for sugar uptake. *E. coli*, for example, uses a chemosmotic transport mechanism for xylose uptake, and it is likely that this mechanism is also active in *Staphylococcus xylosus* and *Staphylococcus saprophyticus* (Jeffries 1983). The chemosmotic uptake mechanism utilizes the transmembrane proton potential to drive uptake so that sugar uptake is accompanied by equimolar symport of protons (H^+). In contrast, the transport systems for **D**-ribose and **D**-glucose in *E. coli* use direct energization and group translocation mechanisms, respectively (Jeffries 1983; Gottschalk 1986). The hydrolysis of ATP is used to provide energy in the direct energization mechanism, whereas the phosphoenolpyruvate hydrolysis serves this purpose in the group translocation mechanism. As a consequence of transport and other metabolic processes, growth rate typically shows Monod-type saturation behavior with respect to sugar concentration. Measured K_m values for xylose catabolism in bacteria range from 4.5-8.0 mMol (Tolan and Finn 1987a,b).

Xylose uptake in yeasts can occur by both facilitated diffusion and active transport processes. In *S. cerevisiae*, a yeast generally recognized not to metabolize xylose (see Batt et al. 1985 for a countering opinion), xylose is transported by facilitated diffusion (Jeffries 1983; Lucas and van Uden 1986). In obligately aerobic *Rhodotorula* sp., transport only takes place under aerobic conditions and for most sugars including xylose and glucose occurs by an active chemosmotic proton symport mechanism (Jeffries 1983; Webb and Lee 1990). There are both low-affinity and high-affinity **D**-xylose carrier proteins in *R. gracilis* (= *R. glutinis*); the high-affinity (low K_m) carrier is repressed during rapid growth but derepressed under starvation conditions. **D**-xylose uptake is competitively inhibited by several hexoses and **D**-glucosamine,

with the higher affinity carrier exhibiting greater specificity. Apparently there is a single common transport system for both **D**-xylose and **D**-galactose, which is subject to catabolite repression in the presence of glucose.

Similar but distinct patterns of sugar transport are observed in *C. shehatae* and *P. stipitis*. Lucas and van Uden (1986) have shown that under starvation conditions at least three separate proton symport systems are active in *C. shehatae*, one for **D**-glucose and **D**-mannose (K_m ~ 0.1 mMol, V_{max} ~ 3.2 mMol/g-h), one for **D**-xylose and **D**-galactose (K_m ~ 1.0 mMol, V_{max} ~ 1.4 mMol/g-h), and another for **L**-arabinose (for which the kinetic constants were not measured). Because of competition for protons, sugars exhibit noncompetitive inhibition towards symport systems other than their own. When cells were grown on either xylose or glucose, however, these sugar symport systems were repressed and a single facilitated diffusion system transported **D**-glucose (K_m ~ 2.2 mMol, V_{max} ~ 2.3 mMol/g-h), **D**-xylose (K_m ~ 126.5 mMol, V_{max} ~ 22.5 mMol/g-h), and **D**-mannose (kinetic constants not measured) such that these sugars competitively inhibited transport of one another. The widely disparate K_m values of this facilitated diffusion system for xylose and glucose are partially compensated for by the tenfold higher V_{max} for xylose uptake.

Both high-affinity and low-affinity symport systems for xylose transport are constitutively expressed in *P. stipitis* (Kilian and van Uden 1988; Does and Bisson 1989). No facilitated diffusion system is present and both symport systems are active in both starved and unstarved cells. The high-affinity system is noncompetitively inhibited by glucose, whereas glucose exerts both substrate inhibition and competitive inhibition on the low-affinity system (Kilian and van Uden 1988). Kinetic constant determinations for the two systems vary widely. Kilian and van Uden (1988) determined kinetic constants of K_m < 0.5 mMol (V_{max} < 1.0 mMol/g-h) and K_m < 2.0 mMol (V_{max} < 3.0 mMol/g-h) for the high-affinity and low-affinity systems, respectively, whereas Does and Bisson (1989) reported the respective K_m values as 0.9 mMol and 380 mMol. Does and Bisson (1989) speculated that this was a result of the different strains and growth conditions used in the two studies.

Xylulose-5-Ⓟ Formation

The pathway for ethanol production from xylulose-5-Ⓟ is largely similar in bacteria, yeast, and fungi. However, the pathways by which xylose is converted to xylulose-5-Ⓟ differ and it is therefore informative to consider in some detail several of the important aspects involved in the formation of this key metabolic intermediate.

Energetics

The energetics of xylose fermentation appear to be less favorable than those for glucose. This is at least partially the result of differences in the formation of the key precursor intermediates for xylose and glucose fermentation, which are, respectively, xylulose-5-Ⓟ and glucose-6-Ⓟ. As discussed by Jeffries (1990), the overall free energy change, ΔG°, for the conversion of xylose to xylulose-5-Ⓟ is probably about two-thirds that for the analogous conversion of glucose to glucose-6-Ⓟ.

Cofactor Requirements of Xylose Reductase

As shown in Figure 2, the pathways by which xylose is converted to xylulose are different in bacteria than in yeast and fungi: bacteria directly isomerize xylose to xylulose using the enzyme xylose isomerase, whereas yeast and fungi use a two-step pathway in which xylose is first reduced by xylose reductase (XR) to xylitol, which is then oxidized to xylulose by xylitol dehydrogenase (XDH). For yeasts and fungi, the

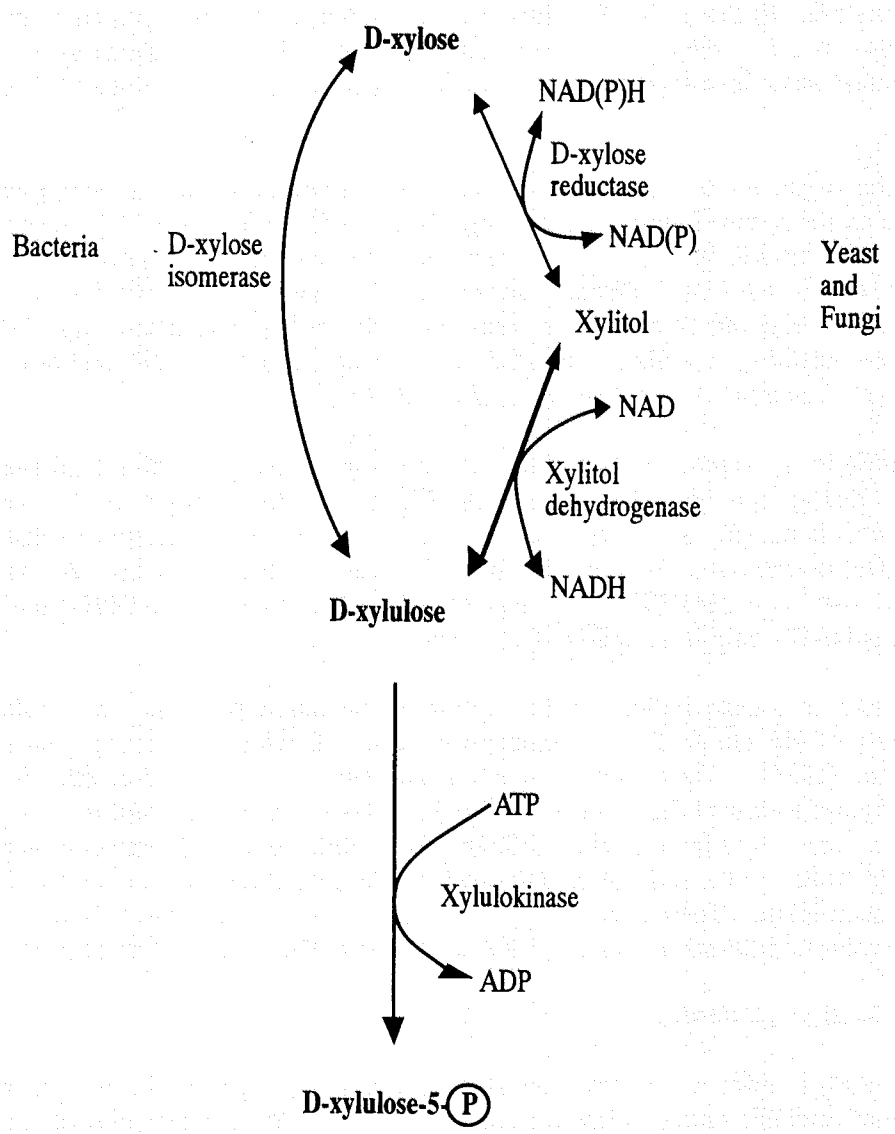

Figure 2. Conversion of D-xylose to D-xylulose-5-phosphate

ability to convert xylose to xylulose-5-Ⓟ is strongly influenced by the coenzyme specificity of XR and XDH enzymes because this affects the ability of the requisite coenzymes to be regenerated. Because mutant strains of *P. stipitis* and *Pa. tannophilus* deficient or lacking in these enzymes are unable to grow or only grow poorly on **D**-xylose (Webb and Lee 1990), the key role of these two enzymes in xylose metabolism by yeast is clear.

Early studies indicated the XR activity required NADPH and NADH for XDH activity. It was believed that the inability to regenerate NADPH and/or NADH created a cofactor imbalance that prevented effective utilization of **D**-xylose. In the mid-1980s, however, a number of studies appeared showing that yeasts such as *P. stipitis* and *Pa. tannophilus* were capable of carrying out reasonably high-yield xylose fermentation. Subsequent investigation demonstrated that forms of NADH-linked XR were present in these yeasts.

Until recently the organism that has been studied most extensively is *Pa. tannophilus*, and early characterizations of XR focused mostly on this organism. Verduyn et al. (1985a), for example, showed that at least two forms of XR existed in *Pa. tannophilus*, one that accepts only NADPH and another that accepts both NADPH and NADH. Ditzelmüller et al. (1985) also isolated two forms of XR from this organism. However, while one form accepted only NADPH, the other accepted only NADH. Although there is still some uncertainty in cofactor specificity, it is clear that both NADH- and NADPH-linked XR activity exists in *Pa. tannophilus* (Lachke and Jeffries 1986).

XR characterization in *P. stipitis* led to similar observations. Verduyn et al. (1985b) isolated a single XR in *P. stipitis* that accepts both NADH and NADPH as cofactor. They also demonstrated that the XR in this organism is actually an aldoreductase, since it is active on a variety of substrates including **DL**-glyceraldehyde, **L**-arabinose, **D**-xylose, **D**-ribose, **D**-galactose, and **D**-glucose. Activity with NADH is about 70% of that with NADPH. NADP+ strongly inhibited both NADPH- and NADH-linked activities, whereas NAD+ only inhibited NADH-linked activity.

The most conclusive evidence indicating that xylose fermentation performance is influenced by the cofactor specificity of XR comes from a comparative study of xylose-fermenting yeasts conducted by Bruinenberg et al. (1984). They showed that ethanol yield and production rate during anaerobic fermentation of xylose followed the level of NADH-linked XR activity (*P. stipitis* > *Pa. tannophilus* > *C. utilis*). A recent study by Ligthelm et al. (1988b) shows that the xylose fermentation performance of *Pa. tannophilus* is related to the ratio of NADH- to NADPH-linked XR and the levels of NADH-linked XR and NAD-linked XDH. Their results suggest the presence of two separate XRs in this organism, because under aerobic conditions induction of NADPH-linked XR precedes that of NADH-linked XR.

Regulation of Xylose Utilization

Webb and Lee (1990) provide a recent review of the regulation of xylose utilization in yeasts. Studies on the regulation of catabolic enzymes involved in xylose utilization have been performed predominantly on yeast and have usually focused on XR and XDH. Growth on **D**-xylose induces XR and XDH activities in both *P. stipitis*, *Pa. tannophilus* (Webb and Lee 1990) and in *C. shehatae* (Alexander et al. 1988b). **L**-arabinose and **D**-galactose also induce these activities in *Pa. tannophilus* (Bolen and Detroy 1985), although xylitol does not (Jeffries 1990).

Many studies of the regulation of xylose utilization have examined how the presence of other sugars influences XR and XDH activities. **D**-glucose and **D**-mannose both repress induction of these enzymes in *P. stipitis* and *Pa. tannophilus*, whereas cellobiose and **D**-galactose do not (Webb and Lee 1990). The behavior with respect to the presence of other sugars differs between these yeasts. For example,**D**-fructose is utilized preferentially to **D**-xylose by *Pa. tannophilus*, but the opposite is true of *P. stipitis*.

A reverse pattern exists for **D**-galactose. *Pa. tannophilus* preferentially utilizes **D**-xylose over **D**-galactose, whereas *P. stipitis* prefers **D**-galactose over **D**-xylose, even though **D**-galactose does not inhibit induction of XR and XDH enzymes. Webb and Lee (1990) state that this type of behavior in *P. stipitis* demonstrates that other processes, such as the regulation of sugar transport or sugar phosphorylation, are also likely to play important roles in determining the overall characteristics of sugar utilization.

In *Pa. tannophilus*, in which regulation has been most extensively studied, it has been demonstrated that induction by **D**-xylose occurs at the level of enzyme expression (Webb and Lee 1990). **D**-glucose and **D**-mannose repress XDH activity more than XR activity, whereas 2-deoxyglucose, a **D**-glucose analogue, represses XDH activity but not XR activity. It is therefore likely that catabolite repression of the XR and XDH enzymes is not under coordinate control in *Pa. tannophilus*. Hexokinase A also appears to play a role in the regulation of catabolite repression in this organism.

The addition of 3%-6% (w/v) **D**-glucose rapidly inactivates **D**-xylose utilization in *C. shehatae, Pa. tannophilus*, and *P. stipitis*, although inactivation is only partial in *C. shehatae* (Webb and Lee 1990). Activity resumes when the **D**-glucose concentration falls, with the threshold values required for reactivation differing between these microorganisms.

Under certain conditions *Pa. tannophilus* and *C. shehatae* can utilize **D**-xylose and **D**-glucose simultaneously. In the case of *Pa. tannophilus*, **D**-glucose is utilized at a somewhat greater rate than **D**-xylose. Although there is simultaneous sugar consumption, the rate of **D**-xylose utilization decreases with time during anaerobic fermentation by *C. shehatae* (Webb and Lee 1990). Experiments at NREL show that under aerobic conditions *P. stipitis* is also able to simultaneously consume **D**-xylose and **D**-glucose, with **D**-glucose consumed somewhat more rapidly than **D**-xylose (Boynton and McMillan 1991, unpublished).

Ethanol and Coproduct Formation

Gottschalk (1986) provides an excellent discussion of microbial metabolism associated with product formation. Yeast and a few bacteria, most notably *Zymomonas mobilis* and *Erwinia amylovora*, contain pyruvate decarboxylase and are therefore able to directly decarboxylate pyruvate to acetaldehyde, the immediate precursor of ethanol. Microorganisms such as *Z. mobilis* and *S. cerevisiae*, which express pyruvate decarboxylase, can ferment hexoses to ethanol at yields approaching the theoretical value of 2 mol ethanol/mol hexose or 0.51 g ethanol/g hexose (1.67 mol/mol pentose or 0.51 g/g pentose). Unfortunately, in their wild-type form these organisms are not capable of fermenting pentoses. Ethanol yields from xylose-fermenting yeasts are generally lower than theoretical and are accompanied to varying degrees by xylitol and/or acetate formation. It is believed that xylitol formation is caused by inhibition of xylitol dehydrogenase by the buildup of NADH in the absence of sufficient respiration. Xylitol formation is not generally observed in yeasts such as *P. stipitis* in which there is appreciable NADH-dependent XR activity. Acetate formation provides ATP by substrate level phosphorylation and is therefore a method of generating additional metabolic energy, which may be useful under physiologically stressed conditions. Although yields remain subtheoretical, coproduct formation in xylose-fermenting yeasts is negligible in many situations. Ethanol yields approaching theoretical have been reported for xylose fermentation by the yeast *P. stipitis* (Skoog and Hahn-Hägerdahl 1990). The high selectivity of ethanol product formation in yeast remains one of the key advantages of using yeasts rather than bacteria or fungi. Coproducts associated with ethanolic fermentation of xylose by bacteria and fungi are listed in Table 2.

Table 2. Nongaseous Coproducts of Ethanolic Xylose Fermentation

Microorganism	Coproducts	Reference
Bacillus macerans	acetate, acetone	Rosenberg et al. (1981)
Clostridium acetobutylicum	acetate, acetone, butanol, butyrate, isopropanol	Saddler et al. (1983) Gottschalk (1986)
Clostridium thermosaccharolyticum	acetate, lactate, 1,2-propanediol	Lynd et al. (1991)
Erwinia chrysanthemi	acetate, formate, lactate	Tolan and Finn (1987a)
Escherichia coli	acetate, formate, lactate, succinate	Bräu and Sahm (1986) Ingram et al. (1987) Ohta et al. (1991)
Fusarium oxysporum	acetate	Singh and Kumar (1991) Singh et al. (1991)
Klebsiella planticola	acetate, 2,3-butanediol, lactate	Tolan and Finn (1987b)
Klebsiella planticola	acetate, formate, lactate	Feldmann et al. (1989)
Klebsiella pneumoniae	acetate, 2,3-butanediol	Saddler et al. (1983)
Paecilomyces lilacinus	acetate, lactate	Mountfort and Rhodes (1991)
Thermoanaerobacter ethanolicus	acetate, lactate, 1,2-propanediol	Carreira et al. (1983) Lacis and Lawford (1988)

As Table 2 illustrates, bacteria and fungi typically carry out mixed product fermentations. In addition to ethanol, products include acetate, acetone, 2,3-butanediol, butanol, butyrate, formate, isopropanol, lactate, 1,2-propanediol (propylene glycol), and/or succinate. The extent and type of coproduct formation varies with microorganism and is affected by medium composition and cultivation conditions. With the exception of the pathway for succinate, which is derived from phosphoenolpyruvate, fermentation products are formed from pyruvate by a variety of enzymatic mechanisms. Ethanol yield during microbial fermentation is largely determined by the selectivity of the pathways by which pyruvate is fermented.

One of the central reasons that the patterns of pyruvate fermentation generally differ in bacteria and fungi from yeasts is that most bacteria and apparently some fungi do not contain pyruvate decarboxylase. This includes most enteric xylose-fermenting bacteria, obligate anaerobes such as *Clostridia* sp., and thermophiles like *Thermoanaerobacter ethanolicus*. Under anaerobic (i.e., fermentative) conditions, organisms such as these use the enzyme pyruvate-formate lyase to split pyruvate into formate and acetyl-coenzyme A (acetyl-CoA) (under aerobic conditions pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase). Thus, in microbes not containing pyruvate decarboxylase there are at least two breakdown products from pyruvate, formate and acetyl-CoA. (Formate is often not detected in chromatographic analyses, as many organisms contain formate-hydrogen lyase, which cleaves formate into hydrogen and carbon dioxide). Acetyl-CoA is converted by acetaldehyde dehydrogenase to acetaldehyde, which serves as the precursor to both ethanol and acetate.

A number of additional routes of pyruvate fermentation are active in organisms not expressing pyruvate decarboxylase. Enteric organisms, for example, produce ethanol by either the mixed acid or the butanediol fermentation pathway. In mixed acid fermentations, lactate is a major coproduct formed from pyruvate through the action of lactate dehydrogenase. In the butanediol fermentation, pyruvate is fermented to 2,3 butanediol via an α-acetolactate intermediate. Another example is the acetone-butanol-ethanol (ABE) fermentation carried out by *Clostridium acetobutylicum* in which acetone and butanol formation proceeds via a phosphoroclastic reaction wherein pyruvate is decarboxylated to acetylphosphate. Examples such as these illustrate that, especially in bacteria, a variety of pathways compete with pyruvate fermentation to ethanol. Pathway selectivity is determined by the metabolic pool of pyruvate and requisite cofactors and the enzyme activities associated with specific pathways, which are under varying degrees of regulation. Apparently, the energetics and/or kinetics of the pyruvate-formate lyase reaction permit the metabolic pool of pyruvate to remain at a level that enables alternative pathways for pyruvate fermentation to effectively compete with ethanol formation. In many instances coproduct formation must accompany ethanol production because it is necessary for cofactor regeneration (e.g., regeneration of NADH). The pathways for pyruvate conversion by mixed acid, butanediol, and ethanolic fermentations are shown in Figure 3.

Genetic Engineering Advances

Recombinant DNA technology is increasingly being used to modify existing microorganisms to improve their ability to ferment xylose to ethanol. Candidate microorganisms for genetic modification are typically lacking only a few key enzymes required for xylose fermentation or only exhibit low activities of such enzymes. Two general strategies, one using facultatively enteric bacterial hosts and the other yeast, have been pursued for constructing improved xylose-fermenting microorganisms. Enteric bacteria such as *E. chrysanthemi, E. coli,* and *Klebsiella sp.* are proficient at assimilating a wide range of sugars, including **D**-xylose, but are not efficient ethanol producers and exhibit relatively low ethanol tolerance in comparison with yeasts. As discussed above, enteric bacteria carry on mixed acid or butanediol fermentations that produce many coproducts in addition to ethanol (ethanol is generally only a minor product). The opposite is largely true for yeasts such as *S. cerevisiae,* which often cannot efficiently assimilate pentoses such as

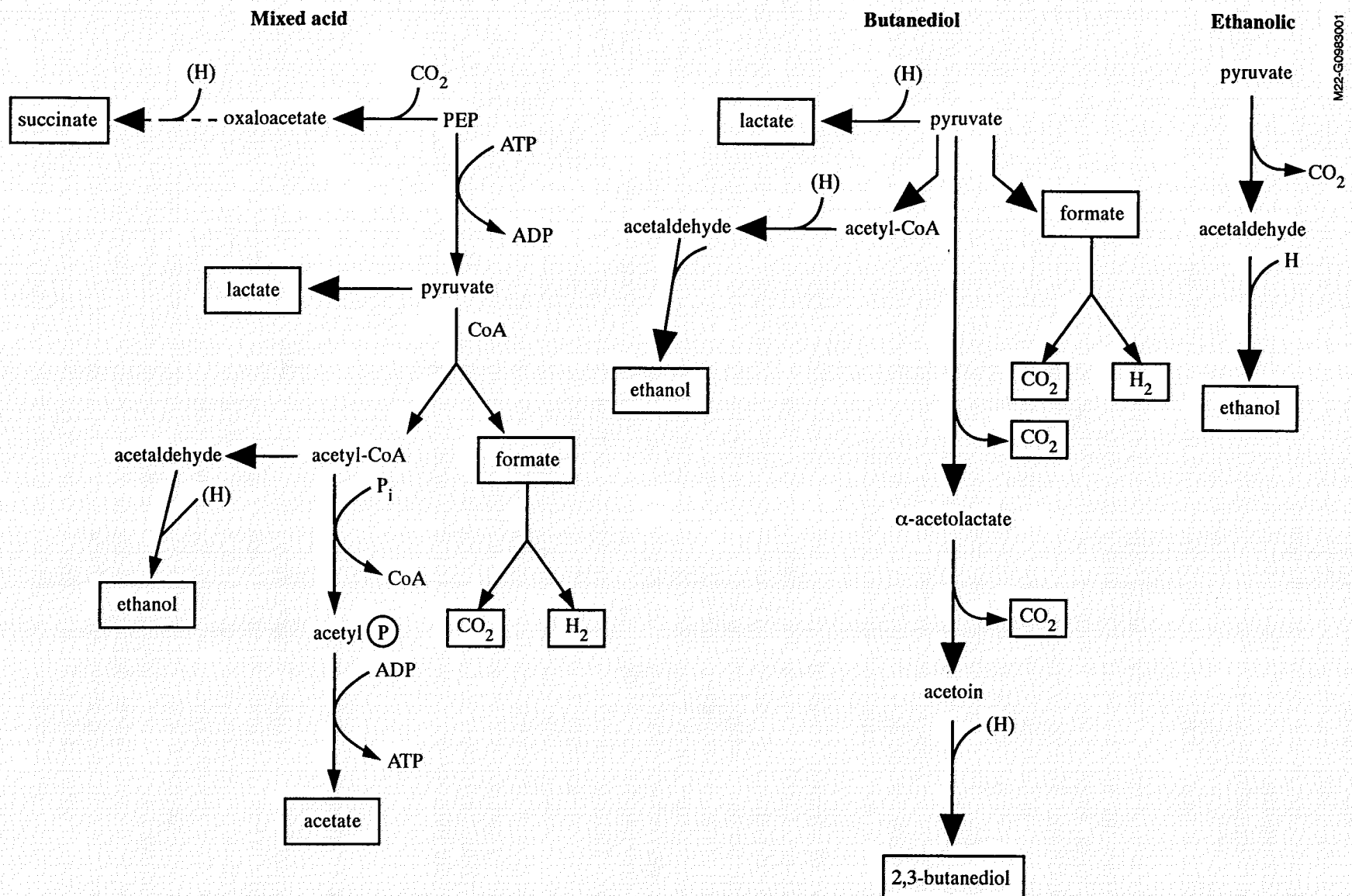

Figure 3. Mixed acid, butanediol, and ethanolic fermentation. Ethanolic fermentation is carried out by yeast and the bacterium *Zymomonas mobilis* (adapted from Gottschalk [1986]).

xylose but offer the advantages of high ethanol yield, negligible coproduct formation, and high ethanol tolerance. As discussed in the following sections, strategies for improving ethanol production by bacteria focus on altering product formation patterns, whereas efforts in yeast concentrate on improving xylose assimilation capabilities.

Bacteria

Bräu and Sahm (1986) were the first to use genetic engineering techniques to improve bacterial ethanol production. They recognized that efficient ethanol-producing microorganisms such as *S. cerevisiae* and *Z. mobilis* could only ferment a limited number of hexose sugars. They reasoned that bacterial ethanol production from renewable feedstocks could be improved by introducing the highly efficient genes involved in ethanol production by *Z. mobilis* into a bacteria able to utilize a broader range of substrates. They achieved this by cloning the *Z. mobilis* ***pdc*** gene coding for pyruvate decarboxylase (PDC) into *E. coli*. During fermentative culture, recombinant *E. coli* harboring the cloned ***pdc*** gene fermented 25 mMol glucose (4.5 g/L) to ethanol at an overall yield of 0.42 g ethanol/g glucose (83% of theoretical), a dramatic improvement over the host organism, which achieved a yield of only 0.06 g/g under otherwise similar conditions. As one would expect, the increase in yield was accompanied by a corresponding decrease in acid coproduct formation (see Table 2).

The success of Bräu and Sahm's work has prompted others to examine this approach. Tolan and Finn (1987a,b) investigated the effect of incorporation of the *Z. mobilis* ***pdc*** gene on the **D**-xylose fermentation performance of *E. chrysanthemi* and *K. planticola*. Expression of the gene in *E. chrysanthemi*, which was selected because of its high ethanol tolerance, increased ethanol yield from 0.21 to 0.44 g/g, again with concomitant reduction in acid coproduct formation (Table 2) (Tolan and Finn 1987a). Unfortunately, the recombinant construct grew at only one-fourth the rate of the wild type and exhibited a markedly lower ethanol tolerance. Yield increased similarly from 0.2 to 0.4 g/g in transconjugated *K. planticola* recombinants carrying a multicopy plasmid expressing the ***pdc*** gene (Tolan and Finn 1987b). Product distribution in recombinant *K. planticola* was strongly influenced by pH, and this was shown to be due to changes in the PDC expression level, which was high at pH 5.6 but dropped off above neutral pH. *Z. mobilis* PDC exhibits maximum activity at pH 6. During fed-batch fermentation of D-xylose at pH 5.8, a final ethanol concentration of 24 g/L was achieved by the recombinant (in comparison to less than 10 g/L by the wild type). The fact that they obtained markedly different results using two closely related enteric organisms led Tolan and Finn (1987b) to conclude that gene insertion causes pleiotropic effects in the host organism that are difficult to predict.

The results of Tolan and Finn (1987b) show that the approach of cloning in the *Z. mobilis* ***pdc*** gene into microbes capable of assimilating xylose is highly promising. However, their results point to several potential problems with this approach. One issue is the formation of coproducts. Although the rate of coproduct (acid) formation was reduced in recombinants expressing the ***pdc*** gene, coproducts, especially acetate, still accumulated to inhibitory levels during prolonged fermentation. It is perplexing that coproduct formation remains a problem to such an extent, since the K_m for pyruvate for PDC is 0.35-0.4 mMol, roughly an order of magnitude less than the K_ms for the other enzymes that act on pyruvate (4.4-7.2 mMol for lactate dehydrogenase and 2.0 mMol for pyruvate-formate lyase) (Ingram and Conway 1988; Neale et al. 1988; Feldmann et al. 1989). Another issue brought up by Tolan and Finn (1987b) is strain stability. Although the growth rate of their transconjugants was roughly 80% of that of the wild type, only 70% of the cells retained the plasmid after batch cultivation on xylose in the absence of antibiotic selection. Most importantly, their results clearly demonstrate the difficulty in predicting *a priori* the performance of a recombinant construct metabolically engineered to achieve a certain end. A recent review by Bailey (1991) illustrates the complexity that pleiotropism brings to the metabolic engineering planning process.

Neale et al. (1988) cloned the ***pdc*** and ***adh*** genes from *Z. mobilis* into *E. coli* and were able to achieve a 0.45 g/g conversion yield of 4% (w/v) glucose into ethanol during anaerobic fermentation. Conversion yields were above 90% for batch fermentation at sugar loadings below 2% (w/v) glucose and 1.2% (w/v) xylose, but the efficiency of conversion dropped off at higher concentrations. It was not clear whether this resulted from ethanol or acid coproduct formation. Feldmann et al. (1989) used a Mu-insertion *K. planticola* mutant lacking the pyruvate-formate lyase as a host organism for cloning studies. Introduction of a plasmid carrying the *Z. mobilis* ***pdc*** gene resulted in a 0.43 g/g ethanol conversion yield from 41 g/L xylose. Similar to Tolan and Finn (1987b), Feldmann et al. (1989) observed a 10% decrease in growth rate upon the introduction and expression of the ***pdc*** gene.

The development by Ingram et al. at the University of Florida of recombinant *E. coli* strains incorporating the *Z. mobilis* ***pdc*** and ***adh*** genes encoding for PDC and alcohol dehydrogenase, respectively (Ingram et al. 1987; Ingram and Conway 1988; Alterthum and Ingram 1989; Ohta et al. 1990; Ingram et al. 1991; Ohta et al. 1991) is most significant. Near theoretical yields of ethanol were achieved by *E. coli* TC4 (pLOI295), a construct harboring the plasmid pLOI295 containing both of the genes, during growth on glucose (Ingram et al. 1987). Ingram and Conway (1988) subsequently showed that despite the fact that the expression level of the plasmid ***pdc*** and ***adh*** gene products in the TC4 strain was excessive, representing up to 17% of the soluble cellular protein, some coproduct formation continued to occur. Ingram's group went on to evaluate the ethanol yield performance of a number of different *E. coli* strains harboring pLOI297, a similar recombinant plasmid containing the *Z. mobilis* ***pdc*** and ***adh*** genes (Alterthum and Ingram 1989). They state that several of the plasmid-free *E. coli* host strains were able to grow on a variety of sugars, including glucose, lactose, mannose, galactose, xylose, and arabinose, although no supporting data are provided. Comparative studies showed ATTC 11303(pLOI297) and ATTC 15224(pLOI297) to be the two recombinant *E. coli* strains showing the best fermentation performance. Yield values obtained by averaging the results of three batch fermentations showed yields of 0.48 and 0.52 g/g for glucose and xylose, respectively. This represents 94% of the theoretical yield for glucose, and more than 100% for xylose. Alterthum and Ingram (1989) hypothesized that the higher than theoretical yield on xylose resulted from the catabolism of complex nutrient components to pyruvate, in addition to pyruvate derived from xylose. Their experiments were carried out in luria broth (LB) medium which, in addition to a sugar carbon source, contained tryptone and yeast extract at levels of 10 and 5 g/L, respectively.

The performance of *E. coli* strain ATTC 11303(pLOI297) has since been optimized to achieve a yield of 0.48 g/g (95% of theoretical) and a maximum productivity of 0.7 g/L-h (gram of ethanol per liter per hour) in batch fermentations of xylose carried out in LB medium (Ohta et al. 1990). A maximum ethanol concentration of 4.8% (w/v) was achieved during fed-batch fermentation of xylose at 30°C; the maximum ethanol concentration fell slightly to 4.6% (w/v) when fermentation was carried out at 35°C. Although the rate declined rapidly and ethanol concentration profiles are only shown for the a period of 30 min, maximum (initial) volumetric productivity in 44 g dry cell weight (DCW)/L and 22 g DCW/L concentrated cell suspensions was 30 and 14 g/L-h, respectively. U.S. patent no. 5,000,000 entitled, "Ethanol production by *Escherichia coli* strains co-expressing *Zymomonas* ***pdc*** and ***adh*** genes," was granted to Ingram, Conway, and Alterthum for this invention (Ingram et al. 1991, assigned to the University of Florida).

In an effort to improve strain stability (antibiotic selection is used in many of their experiments) Ohta et al. (1991) integrated the two genes directly into the *E. coli* chromosome to develop a number of new strains designated KO#. Ethanologenic genes were integrated into the chromosome within the existing pyruvate-formate lyase (***pfl***) gene site to reduce coproduct formation. Initial levels of the expressed enzymes were low, but were improved by subsequent mutation and/or selection to such an extent that new constructs KO4, KO5, and KO20 exhibited glucose and xylose fermentation performance similar to *E. coli* strain ATTC 11303(pLOI297). Succinate production, which remained moderate in the chromosomally

integrated constructs, was reduced by 95% by the introduction of a fumarate reductase (***frd***) mutation. The introduction of a recA mutation, which reduces genetic instability because of homologous recombination, did not improve fermentation performance. Again, yields exceeded theoretical in many experiments, indicative of fermentation on complex LB medium. The maximum volumetric ethanol productivity of the new constructs was about 1.5-1.8 g/L-h during fermentation of 10% (w/v) glucose and about 1.0-1.3 g/L-h for 8% (w/v) xylose. This led Ohta et al. (1991) to conclude that the new chromosomally integrated construct exhibits lower productivity than the plasmid-bearing strain, which achieves maximum volumetric productivities of 1.9 and 1.0 g/L-h during fermentation of 10% (w/v) glucose and 8% (w/v) xylose, respectively. However, these latter values for maximum volumetric productivity are considerably higher than those previously reported. Alterthum and Ingram (1989), for example, cite values of 1.4 and 0.6 g/L-h for fermentation of 10% (w/v) glucose and 8% (w/v) xylose, respectively, by *E. coli* strain ATTC 11303(pLOI297). And Ohta et al. (1990) cite a value of 0.7 g/L-h on 8%(w/v) xylose. It is difficult to fully evaluate their data because of these inconsistencies. Nonetheless, it is clear that these new constructs represent another advance in using genetic engineering to develop xylose-fermenting organisms.

Several authors (Ingram et al. 1987; Feldmann et al. 1989; Ingram and Conway 1988) have suggested that the improved growth characteristics (e.g., increased xylose consumption rates) observed in recombinant constructs containing the *Z. mobilis* ***pdc*** and/or ***adh*** genes are a consequence of reduced acid formation. Recent work by Diaz Ricci et al. (1991) suggests, however, that changes in extracellular pH are relatively unimportant. Rather, changes in the ratio of intracellular NAD(H)/(ADP+ATP), which is a measure of cellular energy charge, appear to be the causative regulatory factor.

Yeast

Progress on applying recombinant DNA technology to develop improved xylose-fermenting yeast has not moved as rapidly as it has for bacteria, although significant breakthroughs have been made recently. The approach has been to use genetic engineering techniques to give ethanologenic yeasts like *S. cerevisiae* the ability to assimilate xylose in the belief that such yeasts would then be able to carry out high-yield, high-productivity ethanolic fermentation of xylose in addition to glucose. The ability of *S. cerevisiae* strains to carry out robust ethanolic fermentation makes them particularly attractive candidates for genetic engineering efforts (Olsson et al. 1992).

Initial attempts to clone the gene from *E. coli* coding for xylose isomerase (XI) into *S. cerevisiae* were unsuccessful (Ho et al. 1983; Sarthy et al. 1987). Sarthy et al. (1987) were able to achieve production of the plasmid-borne gene product, but activity measurements showed the protein to be inactive. They postulated a number of reasons for the lack of proper expression of the *E. coli* enzyme in *S. cerevisiae*, including improper protein folding, different internal pH of bacterial and yeast cells, deleterious posttranslational modifications, inter- or intramolecular disulfide bond formation, and/or the lack of an essential cofactor or metal ion required for XI activity. They were unable to determine which of these effects, if any, was occurring. Chan et al. (1986) were somewhat more successful in cloning the gene for *E. coli* XI into *Schizosaccharomyces pombe*. The recombinant construct exhibited an improved ability to directly ferment xylose to ethanol, although productivity remained low and significant xylitol production occurred. Interpolation of their data indicates a yield of 0.35 g/g (69% of theoretical) and an average volumetric productivity of 0.15 g/L-h during fermentation of 10% (w/v) xylose on a complex yeast extract-malt extract-peptone (YMP) medium.

The direction of more recent efforts has shifted to trying to clone in the key genes responsible for xylose assimilation in yeasts. Each of the enzymes involved in xylose assimilation by yeasts, XR, XDH, and XK, is believed to play an important role in xylose fermentation (Lachke and Jeffries 1986). Deng and Ho

(1990) used recombinant DNA techniques to show that increasing the XK activity in *S. cerevisiae* results in more rapid xylulose fermentation. Several transformation systems for *P. stipitis* have been developed, and progress is being made in cloning xylose assimilation genes from *P. stipitis* into *S. cerevisiae*. Ho et al. (1991) developed a genetic transformation system for the yeast *P. stipitis* that Takuma et al. (1991) utilized to isolate the gene encoding for *P. stipitis* XR. This gene was then cloned into *S. cerevisiae*. Although constitutive expression and XR activity were observed, the recombinant yeast was not able to grow on xylose or ferment xylose to ethanol. Kotter et al. (1990) used a similar approach to clone the *P. stipitis* genes encoding for XR and XDH into *S. cerevisiae*. Although recombinant strains produced very little ethanol, they were able to grow on xylose as the sole carbon source, thus indicating expression and activity of the gene products. Hallborn et al. (1991) transformed *S. cerevisiae* with the gene coding for *P. stipitis* XR and were able to obtain over 95% conversion of xylose to xylitol. A more recent report by Hahn-Hägerdal et al. (1992) indicates that double transformants expressing the both *P. stipitis* XR and XDH are able to grow on xylose (no information on ethanol production was provided).

Efforts continue to improve xylose fermentation using recombinant *S. cerevisiae*. A transformation system for *P. stipitis* has recently been developed that enables the use of auxotrophic selectable markers (Jeffries 1992). Ho (1991a) is planning to overexpress *P. stipitis* genes for XR, XDH and XK in *S. cerevisiae*. Although efforts to clone the gene coding for *E. coli* XI into *S. cerevisiae* have been unsuccessful, both Olsson (1992) and Ho (1991b) put forward the idea of cloning the *Lactobacillus* gene for XI.

As this short survey suggests, significant achievements have been made in the past several years in developing improved xylose-fermenting constructs using genetic engineering techniques. The pace of progress in this area is sufficiently rapid that the construction of new ethanologenic microorganisms capable of fermenting xylose is anticipated in the near future.

Fermentation Studies

Numerous bacteria, yeast, and fungi have been evaluated for their ability to carry out direct high-yield fermentation of xylose to ethanol. This chapter summarizes "best-to-date" performance results reported for promising microorganisms. There is considerably greater information available regarding the cultivation of yeast, particularly for the yeast *C. shehatae, Pa. tannophilus*, and *P. stipitis*, than for bacteria and fungi. Prior to the relatively recent development of recombinant *E. coli* strains, xylose-fermenting yeast were believed to be the best route to high-yield ethanol production. The chapter focuses on studies carried out using xylose as the sole carbohydrate carbon source and reviews available information regarding the influence of medium composition and environmental conditions on xylose conversion performance.

Yeast

The general consensus in the literature is that *C. shehatae, Pa. tannophilus*, and *P. stipitis* are the most promising yeast for direct fermentation of xylose to ethanol; extensive reviews documenting the performance of these yeast have been compiled by Skoog and Hahn-Hägerdal (1988), Prior et al. (1989), and Jeffries (1990). This section summarizes recent research characterizing how the xylose fermentation performance of these yeast is influenced by medium composition, pH, temperature and aeration rate. Table 3 shows a representative sampling of high-yield performance results reported for these yeast.

Medium Composition

The concentration of xylose and the composition of nutrient medium affect xylose conversion performance. Vitamins, trace minerals, and the type of nitrogen source have been shown to be important nutritional factors.

Xylose Concentration

Slininger et al. (1985) examined the influence of the initial xylose concentration on ethanol production during aerobic batch fermentation. The performance of several promising *C. shehatae, P. stipitis,* and *Pa. tannophilus* strains was examined at initial xylose concentrations of 50, 100, 150, and 200 g/L. Only a few of the strains tested utilized all of the xylose supplied when the initial concentration was 100 g/L or greater, and these strains generally exhibited reduced productivities and achieved ethanol yields below 0.35 g/g. Except for *C. shehatae*, xylitol formation increased with increasing xylose concentration. All of the strains of *C. shehatae* and *P. stipitis* were able to ferment 50 g/L xylose to ethanol at yields greater than 0.35 g/g, whereas the yield for *Pa. tannophilus* on 50 g/L xylose was only 0.32 g/g. However, it is inappropriate to directly compare performance data for *Pa. tannophilus*, which was cultivated at 32 °C with that for *C. shehatae* and *P. stipitis*, which were cultivated at 25 °C, since growth rate and ethanol toxicity vary strongly with temperature (see below). Data for the strains that gave the highest yields are presented in Table 3. Where necessary, xylitol concentrations were calculated by multiplying reported xylitol yield by total xylose consumed. As Table 3 shows, most of the *C. shehatae* and *P. stipitis* strains have ethanol volumetric and specific productivities of ~0.3 g/L-h and ~0.2 g/g-h, respectively, although values as high as 0.8-1.0 g/L-h and 0.3-0.5 g/g-h have been reported by du Preezet al. (1986) (see below). Both *C. shehatae* and *P. stipitis* are able to completely ferment 50 g/L xylose to ethanol at yields greater than 0.40 g/g. The data of Slininger et al. (1985) indicate that over the range of xylose concentrations examined, *C. shehatae* achieves somewhat higher yields than *P. stipitis*, although *P. stipitis* is superior in terms of sugar utilization.

du Preez et al. (1986) examined the effect of varying the initial xylose concentration from 10 g/L to 100 g/L on the performance of *C. shehatae* and *P. stipitis* in batch culture at pH 4 and 30°C. Similar to the findings of Slininger et al. (1985), at initial concentrations of 90 g/L or less, both yeast were able to fully utilize supplied xylose. In contrast to the results of Slininger et al. (1985), specific and volumetric productivities showed a strong dependence on xylose concentration at concentrations below 100 g/L. The specific and volumetric productivities of both yeast increased with increasing initial xylose concentration, reaching a maximum at 50 g/L of ~0.40 g/g-h and ~0.90 g/L-h, respectively (see Table 3), and then falling sharply at higher concentrations. *P. stipitis* maintained an ethanol yield greater than 0.40 g/g up to 80 g/L xylose, whereas for *C. shehatae* ethanol yield dropped steadily from 0.39 g/g at 10 g/L xylose to 0.35 at 80 g/L xylose.

Vitamins and Trace Minerals

Jeffries (1985b) shows that *C. shehatae* can carry out ethanol production on defined minimal medium, but that ethanol production rates and yields are greatly stimulated by carrying out fermentation in a medium containing higher levels of vitamins and trace minerals. Biotin and thiamine are necessary vitamins for most xylose-fermenting yeast. Both *Pa. tannophilus* (Jeffries 1985b) and *C. shehatae* require biotin and

Table 3. "Best-to-date" Performance Results for Yeast

Microorganism	Xylose (g/L)	μ (1/h)	Yield (g/g)	Productivity (g/L-h)	Productivity (g/g-h)	Max. Ethanol (g/L)	Other Prod.[a] (g/L)	Xylose Consumed (%)	Medium Type	Reference
C. shehatae NRRL-Y-12856	50		0.45	0.29	0.19	24	1[c]	100	Complex	Slininger et al. (1985)
	100		0.44	0.32	0.16	33	2[c]	75	Complex	
	150		0.40	0.21	0.10	30	0	49	Complex	
	200		0.38	0.18	0.07	26	0	35	Complex	
P. stipitis NRRL-Y-7124	50		0.41	0.28	0.17	20	0	100	Complex	Slininger et al. (1985)
	100		0.42	0.38	0.23	39	1[c]	93	Complex	
	150		0.39	0.30	0.10	52	3[c]	90	Complex	
	200		0.39	0.21	0.06	57	3[c]	72	Complex	
Pa. tannophilus NRRL-Y-2460	50		0.32	0.16	0.08	16	7[c]	100	Complex	Slininger et al. (1985)
	100		0.25	0.13	0.06	24	23[c]	95	Complex	
	150		0.20	0.10	0.03	28	34[c]	95	Complex	
	200		0.18	0.09	0.04	28	38[c]	70	Complex	
C. shehatae CSIR-Y-497	50	0.17[a]	0.37	0.96	0.48	18.5[c]	4[c]	100	Complex	du Preez et al. (1986)
P. stipitis CSIR-Y-633	50	0.15[a]	0.43	0.86	0.30	21.5[c]	0	100	Complex	du Preez et al. (1986)

a = maximum, b = xylitol, c = calculated, d = continuous culture, e = glycerol, f = arabitol, g = ribitol, h = acetic acid

Table 3. "Best-to-date" Performance Results for Yeast (concluded)

Microorganism	Xylose (g/L)	μ (1/h)	Yield (g/g)	Productivity (g/L-h)	Productivity (g/g-h)	Max. Ethanol (g/L)	Other Prod.[a] (g/L)	Xylose Consumed (%)	Medium Type	Reference
C. shehatae CSIR-Y-492	40	0.02[a]	0.37		0.32[a]	15[c]	5.2 1[e], 3[f]	95	Complex	Ligthelm et al. (1988c)
P. stipitis CSIR-Y-633	40	0.08[a]	0.47		0.20[a]	19[c]	2, 0.2[e] 0.4[g]	100	Complex	Ligthelm et al. (1988c)
Pa. tannophilus NRRL-Y-2460	40	0.03[a]	0.28		0.10[a]	11[c]	12 3[e], 2[f]	100	Complex	Ligthelm et al. (1988c)
P. stipitis CBS 6054	50[d]	0.12	0.38	0.35	0.14				Defined	Skoog and Hahn-Hägerdahl (1990)
	50[d]	0.06	0.48	0.50	0.20				Defined	
P. stipitis CSIR-Y-633	50	0.14[a]	0.45	0.92		21.5	0	100	Complex	du Preez and Prior (1985)
C. shehatae CSIR-Y-492	50	0.14[a]	0.36	0.75		20.7	3.2	100	Complex	du Preez and Prior (1985)
C. shehatae CSIR-Y-492	50	0.15	0.36	0.7		16.5	5-8	100	Complex	du Preez et al. (1984)
P. tannophilus NRRL-Y-2460	50	0.14	0.28	0.22		12.2	12 0.5[h]	100	Complex	du Preez et al. (1984)

a = maximum, b = xylitol, c = calculated, d = continuous culture, e = glycerol, f = arabitol, g = ribitol, h = acetic acid

thiamine for growth (Prior et al. 1989). The presence of biotin and thiamine improves the performance of *P. stipitis* markedly, although strains exist that can ferment xylose in the absence of all vitamins (Prior et al. 1989). Recent work by Guebel et al. (1991), however, shows that the supplemental addition of biotin and thiamine to a medium containing 5 g/L yeast extract does not improve the xylose conversion performance of *P. stipitis*, whereas supplemental addition of trace elements does.

Nitrogen Source

The composition of the nitrogen source affects the rate of ethanol production and the ethanol selectivity during anaerobic fermentation of xylose by *Pa. tannophilus* (Jeffries 1983). Nitrate, in particular, inhibits ethanol production under anaerobic conditions, even though it does not affect ethanol production during aerobic conditions. In contrast, cells grown on ammonia and a variety of organic nitrogen sources produce ethanol under both aerobic and anaerobic conditions. Interestingly, the inhibitory effect of nitrate is reduced in the presence of ammonia.

Jeffries (1985b) showed that the type of nitrogen source influences the aerobic xylose conversion efficiency of *C. shehatae*. The effect of three different nitrogen sources, ammonium chloride, peptone, and urea, was compared. Urea and peptone were both superior nitrogen sources relative to ammonium chloride. Urea gave the highest final ethanol concentration, but the xylitol formation rate was also the highest. The use of peptone resulted in a higher final ethanol concentration than when ammonium chloride was used, and it also gave the lowest rate of xylitol formation. Jeffries (1985b) observed that changes in medium composition (i.e., the type of nitrogen source and/or the level of trace mineral components) or culture conditions (i.e., aeration) that permitted more rapid cell growth invariably enabled better ethanol production.

Because ethanol production correlated more closely with cell growth rate than with total cell mass, Jeffries (1985b) concluded that the beneficial effect of nutrient supplementation was due to increased growth rate. The hypothesis that ethanol production by *C. shehatae* increases with increasing growth rate, however, is in conflict with more recent experimental data, as discussed in the section on aeration below.

Verduyn et al. (1985a) observed that the ratio of NADH- to NADPH-linked XR activities in *Pa. tannophilus* is affected by the type of nitrogen source used. The ratio of the activities increases when the fermentation is carried out in a mineral medium rather than in a medium containing yeast extract. The level of NADPH-linked activities is not affected.

Cultivation Conditions

Aeration, pH, and temperature have been shown to have a profound effect on ethanol production by xylose-fermenting yeasts.

Aeration

The level of aeration critically affects the performance of xylose-fermenting yeast. As shown by Bruinenberg et al. (1984), xylose-fermenting yeasts do not ferment xylose to ethanol at appreciable yields under fully aerobic conditions. Rather, under fully aerobic conditions cells grow by respiration alone and exhibit a characteristic aerobic biomass yield of 0.5 g DCW/g xylose (Alexander et al. 1988a). The inability to ferment xylose under highly aerobic conditions may result from the fact that xylose-fermenting yeasts are not subject to a Crabtree-like effect, unlike *S. cerevisiae* growing on glucose. Continuous culture

studies on xylose fermentation by *C. shehatae* have shown that fermentative ethanol production occurs only when growth is physically limited by aeration rather than metabolically limited by finite respiratory capacity (Jeffries et al. 1988). In other words, oxygen limitation is required for ethanol production by xylose-fermenting yeast. Although the presence of some oxygen is required for growth, xylose-fermenting yeast can ferment xylose under strictly anaerobic conditions (du Preez et al. 1984; Skoog and Hahn-Hägerdal 1990).

Unfortunately, very few reports in the literature quantify the level of aeration in terms of oxygen uptake rate, oxygen transfer rate (OTR), or dissolved oxygen concentration. Instead, ill-defined and unquantifiable terms are used such as "fully aerobic," "aerobic," "semi-aerobic," "microaerobic," and "microaerophilic." This leads to confusion when trying to interpret xylose conversion data, since conditions that are defined as "aerobic" by one group may be considered "fully aerobic" or "semi-aerobic" by another. Uniform terminology is needed in this area, and a brief aside to define these terms is warranted.

In terms of mass transfer phenomena, only three levels of aeration exist, aerobic (aeration in excess), oxygen-limited (restricted by rate of aeration), and anaerobic (aeration absent). Aerobic implies that oxygen is not limiting the growth of the culture. Thus, changes in the aeration rate under aerobic conditions do not affect respiratory cell growth. However, in many instances product selectivity is sensitive to changes in the dissolved oxygen level, as is productivity, which may be affected by aeration level even under aerobic conditions. Thus, "fully aerobic" can be interpreted to mean that the dissolved oxygen concentration is at or near 100% air saturation, whereas "aerobic" may also imply this but can also mean that the dissolved oxygen level is below 100% air saturation but well above the critical level at which respiratory cell growth rate is affected (i.e., K_{mO2}). Therefore, changes in the concentration of dissolved oxygen to levels that result in changes in product distribution and/or productivity can occur under "aerobic" conditions. The literature shows that the extent to which changes in dissolved oxygen levels influence product specificity is largely organism and strain dependent. Oxygen-limited, in contrast to aerobic, means that respiratory growth is limited by aeration rate, and in this case the level of dissolved oxygen is near the critical level necessary to support respiratory cellular function. The terms "semi-aerobic," "microaerobic," and "microaerophilic" can be interpreted to mean either relatively low dissolved oxygen aerobic conditions, or oxygen-limited conditions. Anaerobic simply means the absence of aeration; there is no molecular oxygen in an anaerobic system.

The level of dissolved oxygen appears to regulate ethanol production by influencing a variety of enzyme activities associated with xylose assimilation and pyruvate fermentation. Verduyn et al. (1985a) show, for example, that the ratio of NADH- to NADPH-dependent XR activities in *Pa. tannophilus* changes as a function of the extent of aeration. Although the level of NADPH-linked activities is not affected, the ratio of the two activities increases in mineral medium when oxygen limitation is imposed. Ligthelm et al. (1988b) show that XR and XDH are induced more rapidly in *Pa. tannophilus* under aerobic conditions than under anaerobic conditions.

In contrast to the regulatory patterns observed in *Pa. tannophilus*, research on *C. shehatae* (Jeffries et al. 1988; du Preez et al. 1989), and *P. stipitis* (du Preez et al. 1989; Skoog and Hahn-Hägerdal 1990) shows that NAD-dependent XDH and both NADH- and NADPH-dependent XR activities remain relatively constant over a wide range of aeration levels. Rather, Jeffries et al. (1988) show that during xylose fermentation by *C. shehatae* alcohol dehydrogenase (ADH) levels increase by more than fivefold as conditions are changed from fully aerobic to completely anaerobic. Levels of XR and XDH activity and the ratio of NADH/NADPH activity also increase with increasing severity of oxygen-limitation, but by much smaller amounts. Skoog and Hahn-Hägerdal (1990) similarly observe an increase in pyruvate decarboxylase (PDC) activity and a decrease in malate dehydrogenase (MDH) activity (a measure of TCA cycle activity) with increasing oxygen limitation during cultivation of *P. stipitis*.

Table 4 shows performance data for xylose fermentation by *C. shehatae, P. stipitis,* and *Pa. tannophilus* under "microaerophilic" (Slininger et al. 1985) and anaerobic (Ligthelm et al. 1988c) conditions. As a comparison of Table 3 with Table 4 shows, ethanol yields reported by the respective researchers are comparable to those observed during "aerobic" (Slininger et al. 1985) or oxygen-limited (Ligthelm et al. 1988c) fermentation under otherwise similar conditions. However, all strains exhibit decreased specific and volumetric productivities under near or strictly anaerobic conditions except for the higher specific productivity for *Pa. tannophilus* observed by Slininger et al. (1985). The results of Slininger et al. (1985), wherein *C. shehatae* shows a somewhat higher yield but *P. stipitis* utilizes a greater fraction of the supplied xylose, are similar to what they observed under more aerobic conditions. The yield and specific productivity data of Ligthelm et al. (1988c), on the other hand, indicate that *P. stipitis* is more deleteriously affected by anaerobiosis than *C. shehatae.*

Research employing continuous culture techniques has helped to elucidate the influence of aeration on ethanol production in xylose-fermenting yeast. As mentioned above, studies with *C. shehatae* have shown that fermentative ethanol production occurs only when growth is oxygen-limited rather than limited by respiratory capacity (Jeffries et al. 1988). Alexander et al. (1988a) showed that during oxygen-limited growth the ethanol production rate of *C. shehatae* is inversely related to specific growth rate, a finding that is confirmed by recent research carried out by Grootjen et al. (1990) using *P. stipitis.* Their results show that under oxygen-limited cultivation, the specific xylose uptake rate remains equal to the maximum specific transport rate. As the severity of oxygen limitation is increased by reducing the aeration rate, the growth rate of culture falls, making more xylose available for fermentation. Thus, as these researchers observe, the specific fermentation rate increases with decreasing specific aeration rate.

Skoog and Hahn-Hägerdal (1990) carried out a detailed continuous culture study on the effect of oxygen limitation on xylose fermentation by *P. stipitis.* Yield and specific ethanol productivity increased with decreasing OTR at a dilution rate of 0.12 h^{-1}, whereas the overall xylose consumption rate fell. Yield and specific ethanol productivity reached maxima of 0.48 g/g and 0.20 g/g-h, respectively, at a dilution rate of 0.06 h^{-1} and an OTR below 1 mMol/L-h. Under anaerobic conditions, xylose was fermented at a yield of 0.25 g/g and a specific productivity of 0.02 g/g-h. Although no xylitol production was observed at any of the aeration levels investigated, small amounts of glycerol and acetate were produced at OTR levels above 2 mMol/L-h. For comparative purposes, Skoog et al. (1992) carried out a similar study on the effect of oxygenation on glucose fermentations by *P. stipitis.* Results show that increasing oxygen limitation affects the concentrations of intracellular metabolites and key enzymes in a similar manner to xylose fermentations. Skoog et al. (1992) concluded that the similarity of intracellular component concentrations during *P. stipitis* xylose and glucose fermentations indicates that cofactor regeneration requirements do not uniquely limit yield and productivity. The potential for a cofactor imbalance during xylose fermentation primarily results from the two-step reductive/oxidative xylose isomerization pathway which is not used for glucose. The authors suggest that the requirement for small amounts of oxygen (≤1 mMol/L-h) for high-yield ethanol fermentation results from oxygen requirements related to cell growth, proper mitochondrial function, and/or energy generation necessary for xylose transport.

The importance of respiration in regulating xylose-fermenting yeast is also supported by the results of research on the influence of respiratory inhibitors. Addition of respiratory inhibitors during xylose fermentation can be used to increase ethanol production at the expense of xylitol formation and cell growth. Lohmeier-Vogel and Hahn-Hägerdal (1985), for example, showed that the xylose to ethanol conversion yield of *C. tropicalis* doubled from 0.08 to 0.16 g/g in the presence of 0.2 mMol sodium azide under oxygen-limited conditions. Accompanying this change, xylitol yield decreased tenfold from 0.55 to 0.05 g/g. Ligthelm et al. (1988d) studied the effect of the respiratory inhibitors antimycin A (AA),

Table 4. Performance Results for Yeast under Anaerobic or "Microaerophilic" Conditions

Microorganism	Xylose (g/L)	μ (1/h)	Yield (g/g)	Productivity (g/L-h)	Productivity (g/g-h)	Max. Ethanol (g/L)	Other Prod.[a] (g/L)	Xylose Consumed (%)	Medium Type	Reference
C. shehatae NRRL-Y-12856	150		0.42	0.12	0.06	30	6	47	Complex	Slininger et al. (1985)
P. stipitis NRRL-Y-7124	150		0.38	0.22	0.07	45	16	80	Complex	Slininger et al. (1985)
Pa. tannophilus NRRL-Y-2460	150		0.21	0.08	0.06	22	22	69	Complex	Slininger et al. (1985)
C. shehatae CSIR-Y-492	40	0.003[b]	0.41		0.15[b]	16	7.2 0.6[d]		Complex	Ligthelm et al. (1988c)
P. stipitis CSIR-Y-633	40	0.003[b]	0.40		0.02[b]	16	0.2[d] 2.4[e]		Complex	Ligthelm et al. (1988c)
Pa. tannophilus NRRL-Y-2460	40	0.008[b]	0.26		0.07[b]	10[c]	12[c]		Complex	Ligthelm et al. (1988c)
P. stipitis CBS 6054	50	0	0.25	0.20	0.02				Defined	Skoog and Hahn-Hägerdal (1990)

a = xylitol, b = maximum, c = calculated, d = glycerol, e = ribitol

potassium cyanide (KCN), rotenone, and sodium azide on the xylose conversion performance of *P. stipitis* and *Pa. tannophilus*. Of the four respiratory inhibitors examined, only AA stimulated ethanol production by *Pa. tannophilus* and only rotenone stimulated ethanol production by *P. stipitis*. The addition of 0.02 mMol AA increased the specific ethanol productivity and decreased the maximum specific growth rate of *Pa. tannophilus* under both oxygen-limited and anaerobic conditions. Specific productivity increased by 110% over the control under oxygen-limited conditions but only by 40% under anaerobic conditions. Maximum specific growth rates decreased in a corresponding fashion by 20% and 30%, respectively. The addition of 0.5 mMol rotenone increased the specific ethanol productivity and decreased the maximum specific growth rate of *P. stipitis* under both oxygen-limited and anaerobic conditions but to a lesser extent. Specific productivity increased by 40% over the control under oxygen-limited conditions and only by 20% under anaerobic conditions. Rotenone addition reduced the maximum specific growth rate of *P. stipitis* by 30% under oxygen-limited conditions but increased the maximum specific growth rate by 25% under anaerobic conditions.

pH

The intracellular pseudo steady state concentration of **D**-xylose in *Rhodotorula* sp. has been shown to be a reversible function of external pH (Jeffries 1983). Many researchers, for example, Jeffries (1985a) and Alexander et al. (1988b), observe that ethanol production is improved at lower pH. These observations support the hypothesis that xylose symport may limit the rate of xylose utilization, and therefore the rate of ethanol production (see the section on transport). Symport-based sugar uptake systems require a transmembrane proton gradient to drive sugar transport. Since cells tend to operate at a slightly basic internal pH, the transmembrane ΔpH increases when the external pH is reduced (provided that at the lower pH, cellular function and energy metabolism are sufficient to maintain the increased transmembrane ΔpH).

du Preez et al. (1986) examined the effect of pH on the batch fermentation performance of *P. stipitis* and *C. shehatae*. Experiments were carried out at a temperature of 30°C using an initial xylose concentration of 50 g/L xylose. The best performance for *C. shehatae* in terms of growth rate, ethanol yield and specific and volumetric productivity occurred within the pH 3.5-4.5 range. The growth rate (~0.2 h^{-1}) and xylitol yield (~0.05-0.10 g/g) both showed a slight maximum in the range pH 3.5-4.5, whereas the ethanol yield (~0.38 g/g) was insensitive to pH over the entire range examined, pH 2.5-6.5. In contrast, specific and volumetric productivity showed sharp maxima of ~0.45 g/g-h and ~0.95 g/L-h, respectively within the pH 3.5-4.5 range. The performance of *P. stipitis* exhibited a more complex dependence on pH. Whereas growth rate (~0.15 h^{-1}) and specific ethanol productivity (0.30 g/g-h) both showed maxima in the pH 4.5-5.5 range, ethanol yield (~0.43 g/g) and volumetric productivity (~0.85 g/L-h) were maximized within the pH 3.5-4.5 range. Cell yields for both yeast were unaffected by pH.

Temperature

du Preez et al. (1986) also examined the effect of temperature on the batch fermentation performance of *P. stipitis* and *C. shehatae*. Experiments were carried out at pH 4, again using an initial xylose concentration of 50 g/L xylose. Overall performance for both yeast (as measured by specific growth rate, yield, and specific and volumetric productivity) increased with increasing temperature from 18°C to 30°C. Performance fell sharply at higher temperatures ($T \geq 33$°C). *P. stipitis* was less detrimentally affected by increased temperature than *C. shehatae*. The yield for *P. stipitis* remained constant at 0.42 g/g up to 33°C, for example, whereas the yield for *C. shehatae* began to fall at temperatures exceeding 28°C. *C. shehatae* exhibited a linear increase in xylitol production with increasing temperature at temperatures above 22°C. Xylitol production was only observed at 36°C for *P. stipitis*. Interestingly, the growth rate of *P. stipitis*

was also insensitive to increasing temperature. The growth rate of *C. shehatae* reached a maximum at 28°C-30°C, and fell sharply at higher temperature.

du Preez et al. (1987) followed up their earlier study by examining the effect of temperature on the growth rate and ethanol tolerance of *C. shehatae* and *P. stipitis*. The temperature at which cell growth reached a maximum decreased with increasing concentration of exogenously added ethanol. Although cells were able to grow at 46 g/L ethanol over the broader temperature range of 11°-22°C, the optimal temperature for growth of *P. stipitis* fell from 29°-31°C in the absence of ethanol to 14°-16°C in the presence of 46 g/L ethanol. The behavior of *C. shehatae* was similar. Interpolation of their graphical data indicates that the optimal temperature for growth falls from 28°-30°C in the absence of ethanol to 14°-20°C in the presence of 43 g/L ethanol with cells capable of growth at 43 g/L ethanol over the wider temperature range of about 10°-24°C.

Bacteria

The most distinguishing feature of bacteria-based xylose conversion is that there is no requirement for oxygen. A variety of facultatively anaerobic and obligately anaerobic bacteria can ferment xylose to ethanol. As discussed in the section on bacteria, the highest yields and productivities reported to date are obtained using the recombinant strains developed by Ingram et al. However, before discussing the performance of recently developed recombinant bacterial strains, it is useful to review results obtained using nonrecombinant bacteria.

Thermophiles

Research on using nonrecombinant bacteria to carry out direct fermentation of xylose to ethanol has focused on the use of obligately anaerobic thermophilic bacteria. There is a dearth of quantitative information on thermophile performance especially when compared to what is available for yeast. Table 5 shows "best-to-date" performance results reported for *Thermoanaerobacter ethanolicus* and *Clostridium thermosaccharolyticum*, the two thermophiles that have been most extensively investigated (interpolation and direct calculation using reported literature values have been performed where necessary).

The highest yields reported to date are due to Carreira et al. (1983), who observed near theoretical conversion at xylose concentrations below 3% (w/v) using a mutant strain of *T. ethanolicus*. Whereas the parent strain was reported to achieve yields of 0.44 g/g on 1% (w/v) xylose, interpolation indicates that the mutant strain achieved an ethanol yield of 0.5 g/g and a volumetric productivity of 0.12 g/L-h. The final ethanol concentration was 5.1 g/L. Small amounts of acetate and lactate were also formed, however, indicating that the total yield of all products exceeded the theoretical maximum. In more recent studies, Lacis and Lawford (1988,1989) achieved a maximum volumetric productivity of 0.5 g/L-h at a yield of 0.42 g/g in continuous culture of *T. ethanolicus* using a 0.4% (w/v) xylose feed. Their results suggest that the greater than 100% theoretical yield obtained earlier by Carriera et al. (1983) resulted from the fermentation of complex components such as yeast extract.

Although recent work focusing on xylose fermentation has been performed by Lynd et al. (1991), *C. thermosaccharolyticum* has mostly been investigated in the context of mixed culture (DMC) processes. Calculations based on their continuous culture data indicate that *C. thermosaccharolyticum* can ferment xylose to ethanol at a yield of 0.37 g/g and a volumetric productivity above 1 g/L-h but acetate productivity is also high (~0.6 g/L-h). They observed that the ethanol/acetate selectivity ratio increased more than fivefold during transients in which the xylose concentration became non-limiting; however, this

Table 5. "Best-to-date" Performance of Thermophilic Bacteria

Microorganism	Xylose (g/L)	μ (1/h)	Yield (g/g)	Productivity (g/L-h)	Productivity (g/g-h)	Max. Ethanol (g/L)	Other Prod. (g/L)	Xylose Consumed (%)	Medium Type	Reference
T. ethanolicus ATCC 31550 mutant JW-200 Fe(4)	10		0.50[a]	0.12[a]		5.1	0.3[a,b] 0.4[a,c]	100	Complex	Carriera et al. (1983)
T. ethanolicus ATCC-31938	4		0.42	0.50		1.7[a]	0.5[a,b] 0.1[a,c]	100	Complex	Lacis and Lawford (1988, 1989)
C. thermosaccharolyticum HG8	35		0.37	1.03		12.8	7.1[a,b]	97	Complex	Lynd et al. (1991)

a = calculated, b = acetate, c = lactate

increase in product selectivity could not be sustained. It was concluded that unknown regulatory mechanisms involving xylose concentration affect product formation patterns in this organism.

Recombinants

Table 6 shows "best-to-date" performance results reported for recombinant bacteria. The highest yields, volumetric productivities, and final ethanol concentrations have been reported using recombinant *E. coli* strains developed by Ingram et al. (see the section on bacteria). Ingram et al. frequently report yields of greater than 100% of theoretical (Table 6) at least in part because their experiments are carried out in complex medium containing tryptone and yeast extract. Because they usually do not report the concentrations of coproducts, determining the true ethanol yield on xylose from the data they report is difficult, even if approximations are made about the composition of yeast extract. Making assumptions in order to estimate a true yield is unwarranted because uncertainties associated with the numbers they report are large (their experiments have primarily been carried out at the test tube and shake flask scales). This is illustrated by the disparate volumetric productivity data shown in Table 6.

Lawford and Rousseau (1991a,b,c) independently examined the performance of recombinant *E. coli* ATTC 11303 (pLOI297) at the bench scale. Cultivation was carried out batchwise at a temperature of 30°C and a pH of 6.3. Although inoculum was plated on selective medium, no antibiotics were added during fermentations. Using complex medium, 8% (w/v) xylose was fermented at a yield of 0.45 g/g to 36 g/L ethanol with an overall volumetric productivity of 0.47 g/L-h and a maximum specific productivity of 0.27 g/g-h (Lawford and Rousseau 1991a). Yield remained high when cultivation was carried out using minimal defined medium but volumetric and specific productivity dropped significantly (Lawford and Rousseau 1991b). The highest yield reported by these authors is 0.49 g/g, which was achieved during batch fermentation of 2% (w/v) xylose.

Fungi

The use of fungi to carry out xylose fermentation has not been investigated as extensively as has xylose conversion by bacteria and yeast. Most studies are exploratory in nature and carried out at the test tube or shake flask scale. Most investigators use a two-stage process and only monitor ethanol, cell mass, and sugar concentration. In the first stage the fungus is grown aerobically on a carbon source other than xylose (often glucose); anaerobic xylose fermentation is carried out in the second stage. Some researchers perform the entire experiment in a single flask, waiting until the carbon and energy source for growth is exhausted before adding xylose. Others harvest and wash the mycelium and then resuspend it in fresh nutrient solution containing xylose. Since the carbon required for cell mass formation is not considered when yield is calculated, reported ethanol yields in many cases are unrealistically high as a result of this two-stage approach. With this caveat stated, "best-to-date" performance results reported for xylose fermentation by fungi are listed in Table 7.

As Table 7 illustrates, very little research on fungal-based xylose conversion has occurred in the past decade. The most comprehensive studies remain those undertaken in the late 1970s by Wilke et al. examining the performance of *Fusarium oxysporum lini* at the bench scale during both batch and continuous fermentation (Rosenberg et al. 1981). The use of cell recycling increased the cell concentration during continuous fermentation by a factor of 2.6 and caused the volumetric productivity to increase from 0.04 g/L-h to 0.07 g/L-h. It was concluded that these productivities were too low to warrant further investigation and research on the use of *F. oxysporum* was halted.

Table 6. "Best-to-date" Performance Results for Recombinant Bacteria

Microorganism	Xylose (g/L)	μ (1/h)	Yield (g/g)	Productivity (g/L-h)	Productivity (g/g-h)	Max. Ethanol (g/L)	Other Prod. (g/L)	Xylose Consumed (%)	Medium Type	Reference
E. chrysanthemi B374	20		0.37[c]	0.06[c]		7.4	a,f,l	100	Complex	Tolan and Finn (1987a)
K. planticola ATCC 33531 (PDC transconjugants)	5	0.21	0.4	0.28[c]		24	a	98	Complex	Tolan and Finn (1987b)
K. planticola ATCC 33531 (SDF 20)	75		0.237[c]	0.3		17.8	a	55	Defined	Feldman et al. (1989)
E. coli (pZAN4)	12		0.45[c]	0.12[c]		5.4[c]			Complex	Neale et al. (1988)
E. coli ATCC 11303 (pL0I297)	80		0.52	0.6	1.3	42			Complex	Alterthum and Ingram (1989)
E. coli ATCC 11303 (pL0I297)	80		0.48	0.7		39.2			Complex	Ohta et al. (1990)
E. coli ATCC 11303 (pLOI297)	80		0.47	1.0		36.0			Complex	Ohta et al. (1991)
E. coli ATCC 11303 K011 (frd)	80		0.53	1.3		41.6			Complex	Ohta et al. (1991)
E. coli ATCC 11303 (pLOI297)	80		0.45	0.47	0.27[m]	36		100	Complex	Lawford and Rousseau (1991a)
E. coli ATCC 11303 (pLOI297)	64.5		0.43	0.29		28		100	Defined	Lawford and Rousseau (1991b)
E. coli ATCC 11303 (pLOI297)	20.4	0.41	0.49	0.54		9.9		100	Complex	Lawford and Rousseau (1991c)

a = acetate, c = calculated, f = formate, l = lactate, m = maximum

Table 7. "Best-to-date" Performance Results for Fungi

Microorganism	Xylose (g/L)	μ (1/h)	Yield (g/g)	Productivity (g/L-h)	Productivity (g/g-h)	Max. Ethanol (g/L)	Other Prod. (g/L)	Xylose Consumed (%)	Medium Type	Reference
Monilia sp.	50		0.22[c]	0.06[c]		10		40	Complex	Gong et al. (1981)
F. oxysporum lini ATCC 10960	10	0.024[m]	0.41	0.07[m]		3.5	a	98	Defined	Rosenberg et al. (1981)
F. oxysporum VTT-D-801134	50		0.50	0.17		25	a		Complex	Suikho and Enari (1981)
N. crassa NCIM 870	20		0.35	0.04[c]		7			Complex	Deshpande et al. (1986)
Paecilomyces sp. NF1 ATCC 20766	100		0.40	0.24[c]		39.8	a,c		Complex	Wu et al. (1986)
	200		0.37	0.24[c]		73.5	0.5[a,b]	100	Complex	
P. lilacinus IMCP 10835	3.5[x]		0.41 ±0.03				a	60[x]	Defined	Mountfort and Rhodes (1991)
F. oxysporum DSM 840	20		0.20			4.0[c]	5[a,c]		Complex	Singh et al. (1991)
	20		0.46[i]			0.2[c]	0.5[a,c]		Complex	

a = acetate, b = xylitol, i = in presence of respiratory inhibitor, c = calculated, m = maximum, x = xylan

The highest yield reported for fungal conversion of xylose is 0.50 g/g, which Suikho and Enari (1981) obtained for an *F. oxysporum* strain isolated from grain. Their experiments were carried out using the two- stage process outlined above. Average volumetric productivity was 0.17 g/L-h during batch fermentation of 5% (w/v) xylose.

The highest volumetric productivity reported to date was also achieved in a two-stage process. A volumetric productivity of 0.24 g/L-h was measured by Wu et al. (1986) during anaerobic fermentation of xylose by a *Paecilomyces* species. In contrast to what other researchers have reported for yeast and bacteria, yield and volumetric productivity are insensitive to xylose concentration from 5% (w/v) to 20% (w/v) xylose. Furthermore, this fungus was able to completely utilize xylose over this entire concentration range achieving a final ethanol concentration of 73 g/L during fermentation of 200 g/L xylose. Wu et al. reported that ethanol yield was insensitive to pH and temperature, which remained constant from pH 2.2 to pH 7.0 and at both 30°C and 37°C.

Recent research by Singh et al. (1991) shows that the addition of metabolic inhibitors such as sodium azide and 2,4 dinitrophenol (DNP) can be used to increase the ethanol yield during aerobic cultivation of *F. oxysporum*. The ethanol yield achieved during shake flask fermentation of 2% (w/v) xylose increased from ~0.20 g/g to a maximum of 0.46 g/g in the presence of 0.3 mMol sodium azide. Similarly, yield increased from ~0.20 g/g to a maximum of 0.42 g/g when DNP was added at a concentration of 0.2 mMol.

Discussion

A review of the literature shows that a variety of bacteria, yeast, and fungi can ferment xylose to ethanol at yields exceeding 0.40 g/g. Much more performance data are available for yeast than for bacteria and fungi, however, and the recent literature mostly concerns yeast.

Factors Influencing the Performance of Xylose-fermenting Yeast

Aeration appears to be the dominant factor influencing the performance of xylose-fermenting yeast. The composition of nutrient medium, pH, and temperature are secondary factors affecting performance. Optimal medium formulation requires attention to the number of vitamins, the levels of trace minerals, and the type of nitrogen source. High conversion is favored by lower pH and moderate temperature. In microorganisms like xylose-fermenting yeast in which xylose transport can occur by proton symport, intracellular xylose concentration can be influenced by external pH. The observation that ethanol production improves at lower pH may result from the fact that when pH is reduced the transmembrane ΔpH driving force for transport increases. Although higher temperature does not have a pronounced effect on conversion performance *per se*, particularly for *P. stipitis*, the inhibitory effect of ethanol on cell growth increases with increasing temperature so that higher final ethanol concentrations can be achieved by reducing the temperature as ethanol accumulates.

Despite the generalizations that can be made about yeast-mediated xylose conversion, numerous contradictions exist regarding the effects of the different cultivation parameters on overall performance. Slininger et al. (1985) and du Preez et al. (1986), for example, report dissimilar behavior regarding the influence of substrate concentration on the performance of xylose-fermenting yeast. This may reflect the fact that the strains they used differ significantly in their physiological sensitivity to substrate concentration. Differences in experimental design are also likely to have played a role, however, since subtle differences in medium composition and/or cultivation conditions can markedly affect performance. As Tables 3 and 4 show, coproduct formation is another area in which results vary. Different researchers report different levels of polyol (xylitol, glycerol, arabitol, and ribitol) formation under similar conditions.

Although some of these differences can be attributed to physiological differences, such as strain-specific XR and XDH cofactor requirements and xylulokinase regulation, it is evident that factors other than aeration and strain type, perhaps nutritional, are causing different researchers to observe different product formation patterns when investigating similar organisms. Further research is needed to establish a better consensus as to how performance is influenced by changes in oxygenation and nutrient levels.

Xylose-fermenting yeast cannot grow anaerobically, but the reasons for this are not well understood. The ability to ferment xylose under anaerobic conditions shows that the requirement for oxygen is not due to the Kluyver effect observed in some yeast, wherein oxygen is required for sugar utilization (Sims and Barnett 1978). The requirement for oxygen occurs for a different reason than in *S. cerevisiae* which requires oxygen for the synthesis of certain unsaturated lipids (Fiechter et al. 1981; Alexander and Jeffries 1990). Although the addition of yeast extract or ergosterol and oleic acid to growth medium enables *S. cerevisiae* to grow anaerobically, it does not permit anaerobic growth of *Pa. tannophilus* (Maleszka et al. 1982), *C. shehatae* (Alexander et al. 1988a) or *P. stipitis* (Alexander and Jeffries 1990). In addition to lipid synthesis, the poorer energetics of xylose fermentation relative to glucose fermentation may play a role in preventing xylose-fermenting yeast from growing under strict anaerobiosis. As Jeffries (1990) has discussed, the energetics of xylose conversion to xylulose-5-phosphate are less favorable than for formation of glucose-6-phosphate from glucose. Furthermore, the theoretical ATP yield of 1.67 mol ATP/mol xylose fermented is somewhat lower than the 2.0 mol/mol obtained in glucose fermentation. This means that more xylose must be transported to maintain a level of ATP formation comparable to that achieved in glucose fermentation. The requirement for oxygen may indicate that insufficient ATP is produced by substrate level phosphorylation during xylose fermentation. Because acetate formation yields an extra ATP, the fact that the phosphoketolase bypass is operative in some xylose-fermenting organisms supports this conclusion. However, acetate production is not observed to a high degree in xylose-fermenting yeast. The successful use of metabolic inhibitors to block acetate formation in *F. oxysporum* clearly demonstrates that acetate formation is not required for xylose fermentation.

In support of the energetic hypothesis, several researchers have concluded that xylose transport is rate limiting under certain conditions (Kilian and van Uden 1988; Ligthelm et al. 1988a). No facilitated diffusion transport system is found in *P. stipitis*, which also supports the contention that xylose uptake energetics are sufficiently poor to require active transport. However, other yeast such as *S. cerevisiae* and *C. shehatae* are known to use facilitated diffusion to transport xylose (at least under certain conditions) even though *S. cerevisiae* does not utilize xylose. Facilitated diffusion systems for xylose typically exhibit a higher affinity for glucose than for xylose. It has yet to be proven that facilitated diffusion actually plays an important role in xylose transport. In contrast, it is clear that xylose transport by active proton symport is important particularly under starvation conditions. This transport mechanism requires that the cell be sufficiently energized to maintain a transmembrane ΔpH large enough to drive symport. Therefore, the requirement for oxygen may also result from the need to carry out oxidative phosphorylation coupled to ATP synthesis and proton export in order to maintain the transmembrane ΔpH.

While further research is needed to elucidate the importance of oxygen and mitochondrial function in xylose-fermenting yeast, it is interesting to consider why only certain respiratory inhibitors promote ethanol production in these yeast. The respiratory inhibitors KCN (CN^-) and sodium azide (N_3^-) block electron flow between the terminal cytochrome oxidase and molecular oxygen, rotenone inhibits electron transfer within the NADH-Q reductase complex, and AA inhibits the flow of electrons between cytochromes b and c_1 (Stryer 1981). In other words, rotenone and AA act midway in the electron chain, whereas CN^- and N_3^- are terminal inhibitors. The results of Ligthelm et al. (1988d), therefore, suggest that the function of the terminal end of the mitochondrial electron transport chain promotes ethanol production. Ethanol production can, of course, occur under anaerobic conditions in the absence of mitochondrial function but only at a much lower rate and usually at a lower yield than is obtained in the presence of oxygen. It may be that the successful use of respiratory inhibitors results from the fact that

respiratory metabolism is diminished while the level of ATP formation *accompanied by the expulsion of protons* is sufficient to maintain xylose transport. With a constant rate of xylose uptake and a reduced respiratory capacity, more xylose is available for fermentation, which provides an additional means of generating ATP in the presence of reduced respiratory capacity.

Comparison of "Best-to-date" Performance Results

Jeffries (1985a) suggests that xylose conversion processes will be commercially attractive when the following "bench mark" performance values can be achieved:

Performance parameter	Minimum level to achieve for commercialization
Ethanol yield	0.40 g/g
Final ethanol concentration	5% (w/v)
Average volumetric productivity	1.4 g/L-h

Table 8 shows a summary of "best-to-date" performance ranges culled from Tables 3 through 7. A comparison of "best-to-date" performance ranges with Jeffries' bench mark values shows that the main area for improvement is rate or productivity. The best-performing bacteria, yeast, and fungi can all achieve yields greater than 0.4 and final ethanol concentrations approaching 5% (w/v). Except for bacteria, for which the data vary widely, no systems are able to achieve volumetric productivities approaching 1.4 g/L-h. Xylose-fermenting yeast show good potential in terms of yield and final ethanol concentration but remain limited in terms of productivity, especially under anaerobic conditions. Thermophilic bacteria are promising in terms of rate and yield but have only been examined at ethanol concentrations below 1.5% (w/v). Recombinant bacteria exhibit high yield and offer the potential for high anaerobic productivity. However, final ethanol concentrations achieved using recombinant bacteria remain somewhat low. Fungi appear promising in terms of both yield and final ethanol concentration but exhibit lower productivity than either yeast or bacteria.

The yeast *C. shehatae* and *P. stipitis* and the bacterium *E. coli* ATCC 11303(pLOI297) appear the most promising microorganisms examined to date. Relative to *C. shehatae*, *P. stipitis* seems to exhibit somewhat better performance in terms of complete sugar utilization, minimal coproduct formation, and insensitivity to temperature and substrate concentration. Slininger et al. (1985), for example, achieved final ethanol concentrations above 50 g/L with *P. stipitis* but were only able to achieve concentrations of 33 g/L with *C. shehatae*. The results of du Preez et al. (1986) also show superior performance of *P. stipitis* relative to *C. shehatae*. The perplexing insensitivity of the specific growth of *P. stipitis* as temperature was increased over the range 19°-33°C may be the result of increasing maximum growth rate being counteracted by increasing ethanol inhibition. This is supported by the fact that both specific and volumetric productivity exhibit maxima at 28°-30°C.

The work of Ingram et al. (Alterthum and Ingram 1989; Ohta et al. 1990, 1991) demonstrates that recombinant *E. coli* ATCC 11303(pLOI297) exhibits among the most advantageous performance results reported to date. Skepticism accompanied these earlier reports because two of the three yields they reported were greater than theoretical and their volumetric productivity data varied substantially. The results of Lawford and Rousseau (1991a,b,c), however, confirm that recombinant *E. coli* ATCC 11303(pLOI297) can anaerobically ferment xylose to ethanol at high-yield. Significantly, their data also show that good performance can be obtained when the fermentation is carried out in a defined medium.

Table 8. Summary of "Best-to-date" Performance Results

Type of Microorganism	Yield (g/g)	Max. Ethanol %(w/v)	Productivity (g/L-h)	(g/g-h)
Yeast				
Oxygen-limited	0.40-0.48	3-5	0.30-0.90	0.20-0.40
Anaerobic	0.25-0.42	2-4	0.10-0.20	0.02-0.10
Bacteria				
Thermophilic	0.35-0.45	0.1-1.5	0.10-1.00	
Recombinant	0.43-0.49	2-4	0.30-1.30	0.30-1.30
Fungi	0.37-0.46	3-7	0.05-0.20	

Advantages and Disadvantages of Bacteria-based and Yeast-based Processes

The advantages of using a yeast-based (e.g., *P. stipitis*) process for xylose conversion stem from the fact that yeast perform well at low pH and are relatively ethanol tolerant. Both of these factors reduce the probability of bacterial contamination. Another advantage is that antibiotics can be used in cases where pH and/or nutrient excursions are insufficient to eliminate contamination. In addition, yeasts have value as a coproduct which, for example, can be used as a nutrient or protein source to supplement animal feeds. There is also a proven track record of cultivating yeast at the industrial scale.

The primary disadvantages of using a yeast-based process for xylose conversion is the apparent need to supply small amounts of oxygen (≤ 2 mMol/L-h) to achieve high conversion efficiency. Although the rate of oxygen supply can be controlled at low levels relatively easily at the bench or laboratory scale, it is difficult or very expensive to achieve "microaeration" at the industrial scale. A secondary disadvantage of xylose-fermenting yeast is that volumetric productivities are low in comparison to those achievable using bacteria or glucose-fermenting yeast. Ligthelm et al. (1988c), for example, showed that *S. cerevisiae* can ferment glucose at volumetric productivities more than threefold higher than those achieved under comparable conditions in xylose fermentation by the best xylose-fermenting yeast.

Esser and Karsch (1984) provide an excellent review of the merits and weaknesses of bacteria-based ethanol production processes. The major advantages associated with using a bacteria-based (e.g., *E. coli* ATCC 11303 (pLOI297)) process are that no aeration is required and high productivities can be achieved. Potential disadvantages include higher sensitivity to ethanol inhibition, increased probability of contamination caused by operation at higher pH, and loss of productivity because of plasmid instability during prolonged operation (which can be overcome by chromosomal integration). The uncertainty concerning successful large-scale fermentation of bacteria is greater than that associated with yeast. Although this makes it difficult to speculate on the final outcome of attempting large-scale cultivation of a bacterium, it is certain that the startup and associated learning curve for successfully operating such a process will be greater than a similar effort undertaken with yeast (except for the aeration requirement for xylose-fermenting yeast, which presents unique difficulties). The use of a recombinant bacterium at large-

scale may also involve regulatory obstacles and containment requirements that would be avoided by using a wild-type organism. However, a recombinant with antibiotic resistance would enable selection pressure to be imposed to eliminate contamination when necessary.

There are several potential advantages of using xylose-fermenting yeast that are related more to the efficacy of an overall biomass to ethanol process than to xylose conversion *per se*. Some yeasts and many fungi are able to ferment a broad range of sugars and polyols to ethanol. *Pa. tannophilus*, for example, can ferment glycerol to ethanol (Maleszka et al. 1982). *P. stipitis* is reported to be capable of fermenting cellobiose, and some strains exhibit xylanolytic ability (Prior et al. 1989). These are potentially important advantages to the use of yeast rather than recombinant *E. coli* in a xylose conversion process developed in the context of an overall biomass to ethanol scheme. Moreover, the economic analysis of Hinman et al. (1989) indicates that yield and final ethanol concentration rather than production rate are the key to cost-effective xylose conversion in the context of an integrated biomass to ethanol conversion process. Thus, the relatively low productivities of yeast-based systems may not be as disadvantageous as they appear.

Key Issues

Despite substantial progress in the past decade, considerable research remains to be done to develop economic large-scale xylose fermentation processes. Some of the key issues that must be addressed are listed below.

Nutrient Requirements (Including Aeration)

Economic large-scale ethanol production requires minimizing nutrient (i.e., medium and aeration) costs. Thus, the determination of performance on cost-effective minimal or defined medium composition is essential. The summaries of "best-to-date" performance data (Tables 3-7) show, for example, that most data are obtained using rich, complex media. Xylose is not the sole carbon source in complex media so many reported yield values are artificially high. Moreover, performance on minimal cost-effective medium is not likely to be as good as that observed on nutrient-rich medium (as the results of Lawford and Rousseau [1991b] show). The cost of supplying microaeration at an industrial scale must also be considered when assessing the economics of yeast-based xylose conversion processes.

Performance on Real Hydrolyzates

Real hydrolyzates often contain both inhibitory components like acetate and phenolic compounds and sugars other than **D**-xylose such as **D**-glucose and **L**-arabinose. Growth and fermentation characteristics of xylose-fermenting organisms cultivated in real hydrolyzates must be assessed. It is unclear that the highest values reported to date for conversion of pure xylose can be achieved during fermentation of real hydrolyzates (Schneider 1989). Similarly, medium requirements are likely to be influenced by the presence of inhibitory components. Studies might best be broken down into three aspects: 1) performance on sugar mixtures, 2) performance in the presence of inhibitory components found in real hydrolyzates, and 3) performance on real hydrolyzates containing mixtures of sugars and inhibitory components.

Performance and Stability during a Prolonged Culture

Very few studies have been undertaken to characterize performance during extended fed-batch and/or continuous fermentation. The feasibility of developing a large-scale process requires robust long-term operation, which must first be demonstrated at the bench scale. Long-term stability is a particular concern if a recombinant organism is to be used. In this case, a careful evaluation of genetic stability must be

carried out early in the development of the process. Large-scale operation, even if conducted batchwise, requires that an inoculum culture be cultivated for many more generation cycles than typically encountered at the bench scale.

Development of Improved Xylose-fermenting Microorganisms using Recombinant DNA Technology

Recent developments are remarkable. Even so, further progress in the development and characterization of recombinant xylose-fermenting microorganisms is needed. As discussed above, there are potential drawbacks to the use of recombinant *E. coli*. Most notably, there is a larger potential for contamination during prolonged (e.g., continuous) large-scale cultivation at nearly neutral pH than with a yeast-based system, which would operate at lower pH. This motivates the need to develop recombinant microorganisms, such as yeast, for which the potential for contamination is reduced.

Olsson et al. (1992) observed that *S. cerevisiae* already ferments hexoses well at low pH and in non-detoxified substrates and thus is an attractive host for genetic engineering approaches to develop improved xylose-fermenting organisms. Current efforts to make a recombinant *S. cerevisiae* capable of fermenting xylose include cloning in either the *Lactobacillus brevis* gene encoding for XI or the genes encoding for XR and XDH in *P. stipitis*. Recent progress in the development of cloning systems for *Lactobacillus* species increases the likelihood that a *Lactobacillus* XI can be expressed in *S. cerevisiae* (Posno et al. 1991). It is also likely that further advances in cloning the genes for xylose assimilation from *P. stipitis* into *S. cerevisiae* will be made.

Medium requirements, genetic stability, and environmental acceptability are other factors that must be considered early on when attempting to develop recombinant species for industrial xylose fermentation processes. Recombinant microorganisms developed in the laboratory typically have complex (i.e., expensive) nutritional requirements. Recombinant constructs are also often unstable in long-term culture and may quickly revert to their wild phenotype when grown on minimal defined medium in the absence of antibiotic selection.

Development of Improved Xylose-fermenting Microorganisms by Adaptation

The results of Parekh et al. (1986), although not yet reproduced by independent laboratories, suggest that strain adaptation offers tremendous potential for improving xylose conversion performance. Research needs to be undertaken to explore the potential for using adaptation to increase the ethanol tolerance of xylose-fermenting microorganisms. Adaptation may also be useful for developing organisms with improved tolerance to inhibitory components in pretreatment hydrolyzate. Successful adaptation would result in the ability to achieve higher final ethanol concentrations.

Isolation of Improved Xylose-fermenting Organisms from Nature

Within the last 12 years, it has been discovered that many wild-type yeast strains, notably *C. shehatae* and *P. stipitis*, and some fungi, such as *Paecilomyces* species, are capable of direct high-yield fermentation of xylose to ethanol. It is possible that with further efforts wild-type microorganisms with improved performance characteristics can be identified. Isolation and screening programs devised to identify improved xylose-fermenting microorganisms should continue to be pursued.

Techniques for Providing Microaeration at Large Scales

Developing an economic process based on yeast will require that cost-effective techniques for supplying low levels of aeration ($\leq$ 2 mMol/L-h) be developed.

Potential for Cell Immobilization and/or Cell Recycling Techniques to Improve Productivity

Innovative approaches may be required to improve the volumetric productivity of yeast-based processes. As recently pointed out by Grootjen et al. (1990), the fact that the specific substrate uptake rate reaches a maximum under oxygen-limited growth (which is required for high-yield ethanol production) means that the only way to increase productivity further is to increase cell concentration. Cell immobilization has been suggested as a technique for increasing productivity to achieve acceptable yields and final ethanol concentration (Slininger et al. 1985). Recent efforts to realize increased productivity through cell recycling and/or multi-staged continuous culture have, however, been unsuccessful (Alexander et al. 1987; Sreenath and Jeffries 1987; Alexander et al. 1988c).

Fundamental Aspects of D-xylose Metabolism

A number of important questions about xylose metabolism remain unanswered. For example, does the catabolite repression observed in yeast at high xylose concentration and in the presence of mixed sugars occur at the transcriptional or translation level and what role do other regulators such as hexokinase A play (Webb and Lee 1990)? Why are a number of key enzymes equally important during fermentative metabolism whereas only xylulokinase activity is critical for aerobic xylose assimilation and cell growth (Lachke and Jeffries 1986)? Other questions include: Why do xylose-fermenting yeast require oxygen for growth? What features cause xylose transport to be limiting in yeast under aerobic conditions but not limiting under anaerobic conditions? How do thermophilic bacteria regulate product selectivity? How do different microorganisms metabolize mixtures of sugars? How is sugar metabolism regulated? Induced? Repressed?

Answers to questions such as these will lead to strategies for improving xylose fermentation performance. For example, if the reason that yeast require small amounts of oxygen can be determined, it may be possible to devise methods for circumventing or eliminating this requirement.

Research at NREL

Despite the enormous advances that have been made, many issues must be resolved before a thorough technoeconomic examination of large-scale xylose to ethanol production can be made. In particular, performance on real pretreatment hydrolyzates containing a mixture of sugars and inhibitory components must be established, and the requirements for additional nutrients (including oxygen) quantified. The literature that has been published on the conversion of pure xylose, sugar mixtures, and real hydrolyzates does not provide sufficient data on byproduct concentrations and/or carbon dioxide evolution for elemental carbon balances to be made to check the consistency of calculated performance values.

At NREL, a state-of-the-art fermentation monitoring system is being constructed that will be used to reliably and quantitatively evaluate microorganisms being considered for use in large-scale xylose conversion processes. This system will enable NREL researchers to carry out elemental carbon balancing in real time so that ethanol yield and productivity can be quantified as a function of operating conditions such as aeration rate, xylose concentration, pH, and temperature. This system will make it possible to carry out rigorous back-to-back evaluations of xylose-fermenting organisms. The results of such studies will be used to direct development of pilot-scale xylose to ethanol fermentation processes.

Conclusions

Tremendous advances have been made in the last several years in the understanding of xylose metabolism and in the identification and development of strains with improved xylose fermentation characteristics. The successful construction of recombinant bacterial and yeast strains exhibiting improved xylose fermentation performance characteristics arguably represents the single most significant development in xylose conversion research in the past several years.

A survey of the numerous microorganisms capable of directing fermenting xylose to ethanol indicates that wild-type yeast and recombinant bacteria offer the best overall performance in terms of high-yield, final ethanol concentration, and volumetric productivity. The best performing bacteria, yeast, and fungi can achieve yields greater than 0.4 g/g and final ethanol concentrations approaching 5% (w/v). Productivities remain low for most yeast and particularly for fungi but volumetric productivities exceeding 1.0 g/L-h have been reported for xylose-fermenting bacteria. In terms of wild-type microorganisms, strains of the yeast *P. stipitis* show the most promise in the short term for direct high-yield fermentation of xylose without byproduct formation. The major disadvantage to the use of *P. stipitis* is that very low levels of aeration must be provided to achieve optimal performance. Recombinant *E. coli* ATTC 11303 (pLOI297) is the bacterium that exhibits the most favorable performance characteristics reported to date. The disadvantages to the use of this organism are its unproven genetic stability, the higher probability of contamination at nearly neutral pH, and the fact that regulatory obstacles may exist regarding its use at a large scale. Although not researched to the same degree as yeast and recombinant bacteria, several fungi and thermophilic bacteria show long-term promise and merit further evaluation.

The current pace of developments in the xylose conversion field is rapid. A considerable body of literature already exists on the performance of xylose-fermenting yeast. Data on the performance of recombinant *E. coli* are also accumulating quickly. Genetic engineering efforts aimed at constructing a recombinant *S. cerevisiae* strain capable of high-yield xylose fermentation are close to fruition and the construction of improved bacterial strains is also under way. With the many improvements that are being made in realizing efficient high-yield xylose fermentation, the successful development of large-scale processes should be possible within the decade.

References

Alexander, M.A.; Jeffries, T.W. (1990). "Respiratory Efficiency and Metabolite Partitioning as Regulatory Phenomena in Yeasts." *Enzyme and Microbial Technology* (12); pp. 2-19.

Alexander, M.A.; Chapman, T.W.; Jeffries, T.W.; (1988a). "Xylose Metabolism by *Candida shehatae* in Continuous Culture. *Applied Microbiology and Biotechnology* (28); pp. 478-486.

Alexander, M.A.; Yang, V.W.; Jeffries, T.W. (1988b). "Levels of Pentose Phosphate Pathway Enzymes from *Candida shehatae* Grown in Continuous Culture." *Applied Microbiology and Biotechnology* (29); pp. 282-288.

Alexander, M.A.; Chapman, T.W.; Jeffries, T.W. (1988c). "Continuous Xylose Fermentation by *Candida shehatae* in a Two-stage Reactor." *Applied Biochemistry and Biotechnology* (17); pp. 221-229.

Alterthum, F.; Ingram, L.O. (1989). "Efficient Ethanol Production from Glucose Lactose and Xylose by Recombinant *Escherichia coli.*" *Applied and Environmental Microbiology* (55:8); pp. 1943-1948.

Bailey, J.E.; Shanks, J.V. (1991). "^{31}P NMR and ^{13}C NMR Studies of Recombinant *Saccharomyces cerevisiae* with Altered Glucose Phosphorylation Activities." *Bioprocess Engineering* (6); pp. 273-284.

Bailey, J.E. (1991). "Toward a Science of Metabolic Engineering." *Science* (252); pp. 1668-1675.

Batt, C.A.; Carvallo, S.; Easson, D.D.; Akedo, M. Jr.; Sinskey, A.J. (1985). "Direct Evidence for a Xylose Metabolic Pathway in *Saccharomyces cerevisiae.*" *Biotechnology and Bioengineering* (28); pp. 549-553.

Bolen, P.L.; Detroy, R.W. (1985). "Induction of NADPH-linked **D**-xylose Reductase and NAD-linked Xylitol Dehydrogenase Activities in *Pachysolen tannophilus* by D-xylose L-arabinose or **D**-galactose." *Biotechnology and Bioengineering* (27); pp. 302-307.

Bräu, B.; Sahm, H. (1986). "Cloning and Expression of the Structural Gene for Pyruvate Decarboxylase of *Zymomonas mobilis* in *Escherichia coli.*" *Archives of Microbiology* (144); pp. 296-301.

Bruinenberg, P.M.; de Bot, P.H.M.; van Dijken, J.P.; Scheffers, W.A. (1983). "The Role of Redox Balances in the Anaerobic Fermentation of Xylose by Yeasts." *European Journal of Applied Microbiology and Biotechnology* (18); pp. 287-292.

Bruinenberg, P.M.; de Bot, P.H.M.; van Dijken, J.P.; Scheffers, W.A. (1984). "NADH-linked Aldose Reductase: the Key to Anaerobic Alcohol Fermentation of Xylose by Yeasts." *European Journal of Applied Microbiology and Biotechnology* (19); pp. 256-260.

Carreira, L.H.; Wiegel, J.; Ljungdahl, L.G. (1983). "Production of Ethanol from Biopolymers by Anaerobic Thermophilic and Extreme Thermophilic Bacteria: I. Regulation of Carbohydrate Utilization in Mutants of *Thermoanaerobacter Ethanolicus.*" *Biotechnology and Bioengineering Symposium* (13); pp. 183-191.

Chaing, C.; and Knight, S.G. (1960). "Metabolism of **D**-xylose by Moulds." *Nature* (188); pp. 79-81.

Chan, E-C.; Ueng, P.P.; Chen, L. (1986). "**D**-xylose Fermentation to Ethanol by *Schizosaccharomyces pombe* Clones with Xylose Isomerase Gene." *Biotechnology Letters* (8:4); pp. 231-234.

Deng, X.X.; Ho, N.W.Y. (1990). "Xylulokinase Activity in Various Yeasts including *Saccharomyces cerevisiae* Containing the Clones Xylulokinase Gene." *Applied Biochemistry and Biotechnology* (24/25); pp. 193-199.

Deshpande, V.; Keskar, S.; Mishra, C.; Rao, M. (1986). "Direct Conversion of Cellulose/Hemicellulose to Ethanol by *Neurospora crassa.*" *Enzyme and Microbial Technology* (8); pp. 149-152.

Diaz Ricci, J.C.; Hitzmann, B.; Bailey, J.E. (1991). "*In vivo* NMR Analysis of the Influence of Pyruvate Decarboxylase and Alcohol Dehydrogenase of *Zymomonas mobilis* on the Anaerobic Metabolism of *Escherichia coli.*" *Biotechnology Progress* (7); pp. 305-310.

Ditzelmüller, G.; Kubicek-Pranz, E.M.; Röhr, M.; Kubicek, C.P. (1985). "NADPH-specific and NADH-specific Xylose Reduction is Catalyzed by Two Separate Enzymes in *Pachysolen tannophilus.*" *Applied Microbiology and Biotechnology* (22); pp. 297-299.

Does, A.L.; Bisson, L.F. (1989). "Characterization of Xylose Uptake in the Yeasts *Pichia heedii* and *Pichia stipitis.*" *Applied and Environmental Microbiology* (55:1); pp. 159-164.

du Preez, J.C.; van Driessel, B.; Prior, B.A. (1989). "Effect of Aerobiosis on Fermentation and Key Enzyme Levels during Growth of *Pichia stipitis Candida shehatae* and *Candida tenuis* on **D**-xylose." *Archives of Microbiology* (152); pp. 143-147.

du Preez, J.C.; Bosch, M.; Prior, B.A. (1987). "Temperature Profiles of Growth and Ethanol Tolerance of the Xylose Fermenting Yeasts *Candida shehatae* and *Pichia stipitis.*" *Applied Microbiology and Biotechnology* (25); pp. 521-525.

du Preez, J.C.; Bosch, M.; Prior, B.A. (1986). "The Fermentation of Hexose and Pentose Sugars by *Candida shehatae* and *Pichia stipitis.*" *Applied Microbiology and Biotechnology* (23); pp. 228-233.

du Preez, J.C.; Prior, B.A. (1985). "A Quantitative Screening of some Xylose-fermenting Yeast Isolates." *Biotechnology Letters* (7:4); pp. 241-246.

du Preez, J.C.; Prior, B.A.; Monterio, A.M.T. (1984). The Effect of Aeration on Xylose Fermentation by *Candida shehatae* and *Pachysolen tannophilus*: a Comparative Study." *Applied Microbiology and Biotechnology* (19); pp. 261-266.

Esser, K.; Karsch, T. (1984). "Bacterial Ethanol Production: Advantages and Disadvantages." *Process Biochemistry* (19); pp. 116-120.

Evans, C.T.; Ratledge, C. (1984). "Induction of Xylulose-5-phosphate Phosphoketolase in a Variety of Yeasts Grown on **D**-xylose: the Key to Efficient Xylose Metabolism." *Archives of Microbiology* (139); pp. 48-52.

Feldmann, S.; Sprenger, G.A.; Sahm, H. (1989). "Ethanol Production from Xylose with a Pyruvate-formate-lyase Mutant of *Klebsiella planticola* carrying a Pyruvate-decarboxylase Gene from *Zymomonas mobilis.*" *Applied Microbiology and Biotechnology* (31); pp. 152-157.

Fiechter, A.; Fuhrmann, G.F.; Käppeli, O. (1981). "Regulation of Glucose Metabolism in Growing Yeast Cells. *Advances in Microbial Physiology* (22); pp. 123-183.

Gong, C-S.; Maun, C.M.; Tsao, G.T. (1981). "Direct Fermentation of Cellulose to Ethanol by a Cellulolytic Filamentous Fungus *Monilia sp.*" *Biotechnology Letters* (3:2); pp. 77-82.

Gottschalk, G. (1986). *Bacterial Metabolism: Second Ed.* New York: Springer-Verlag.

Grootjen, D.R.J.; van der Lans, R.G.J.M.; Luyben, K.Ch.A.M. (1990). "Effects of the Aeration Rate on the Fermentation of Glucose and Xylose by *Pichia stipitis* CBS 5773." *Enzyme and Microbial Technology* (12); pp. 20-22.

Guebel, D.V.; Cordenons, A.; Nudel, B.C.; Giulietti, A.M. (1991). "Influence of Oxygen Transfer Rate and Media Composition on Fermentation of D-xylose by *Pichia stipitis* NRRL Y-7124." *Journal of Industrial Microbiology* (7); pp. 287-292.

Hahn-Hägerdal, B.; Lindén, T.; Senac, T.; Skoog, K. (1991). "Ethanolic Fermentation of Pentoses in Lignocellulose Hydrolysates." *Applied Biochemistry and Biotechnology* (28/29); pp. 131-144.

Hallborn, J., M. Walfridsson, U. Airaksinen, H. Ojamo, B. Hahn-Hägerdal, M. Penttilä, and S. Keränen. (1991). "Xylitol Production by Recombinant *Saccharomyces Cerevisiae.*" *Bio/Technology,* 9:1090-1095.

Hinman, N.D.; Wright, J.D.; Hoagland, W.; Wyman, C.E. (1989). "Xylose Fermentation: an Economic Analysis." *Applied Biochemistry and Biotechnology* (20/21); pp. 391-401.

Ho, N.W.Y.; Stevis, P.; Rosenfeld, S.; Huang J.J.; and Tsao, G.T. (1983). "Expression of the *E. coli* Xylose Isomerase Gene by a Yeast Promoter." *Biotechnology and Bioengineering Symposium* (13); pp. 245-250.

Ho, N.W.Y.; Petros, D.; Deng, X.X. (1991). "Genetic Transformation of Xylose-Fermenting Yeast *Pichia stipitis.*" *Applied Biochemistry and Biotechnology* (28/29); pp. 369-375.

Ho, N.W.Y. (1991a). "Cloning and Overexpression of Xylose Reductase and Xylulokinase Gene in *Saccharomyces cerevisiae* for Improving its Xylose Fermentation." Paper presented at 13th Symposium on Biotechnology for Fuels and Chemicals, Colorado Springs, Colorado, May 6-10, 1991.

Ho, N.W.Y. (1991b). "Genetic Modification of Yeast for Improving its Xylose Fermentation." A research proposal submitted to the National Renewable Energy Laboratory, Golden, Colorado.

Horecker, B.L. (1962). *Pentose Metabolism in Bacteria.* New York: Wiley.

Ingram, L.O.; Conway, T.; Alterthum, F. (1991). "Ethanol Production by *Escherichia coli* Strains co-expressing *Zymomonas* PDC and ADH Genes." *United States Patent* No. 5,000,000.

Ingram, L.O.; Conway, T. (1988). "Expression of Different Levels of Ethanologenic Enzymes from *Zymomonas mobilis* in Recombinant Strains of *Escherichia coli.*" *Applied and Environmental Microbiology* (54:2); pp. 397-404.

Ingram, L.O.; Conway, T.; Clark, D.P.; Sewell, G.W.; Preston, J.F. (1987). "Genetic Engineering of Ethanol Production in *Escherichia coli*." *Applied and Environmental Microbiology* (53:10); pp. 2420-2425.

Jeffries, T.W. (1992). "Strain Selection and Genetic Improvement of Xylose Fermenting Yeasts." Presentation at the Eighth International Symposium on Yeasts. Atlanta, Georgia, August 23-28.

Jeffries, T.W. (1990). "Fermentation of D-xylose and Cellobiose." *Yeast: Biotechnology and Biocatalysis;* Verachtert, H., De Mot, R., eds. New York: Marcel Dekker; pp. 349-394.

Jeffries, T.W.; Cullen, D.; Alexander, M.A. (1988). "Regulation of Alcohol Dehydrogenase in Xylose and Glucose Fermentations." *Chimica Oggi* (Oct); pp. 23-27.

Jeffries, T.W. (1985b). "Effects of Culture Conditions on the Fermentation of Xylose to Ethanol by *Candida shehatae*." *Biotechnology Bioengineering Symposium* 15; pp. 149-166.

Jeffries, T.W. (1985a). "Emerging Technology for Fermenting D-xylose." *Trends in Biotechnology* (3); pp. 208-212.

Jeffries, T.W. (1983). "Utilization of Xylose by Bacteria Yeasts and Fungi." *Advances in Biochemical Engineering-Biotechnology* (27); pp. 1-32.

Kilian, S.G.; van Uden, N. (1988). "Transport of Xylose and Glucose in the Xylose-Fermenting Yeast *Pichia stipitis*." *Applied Microbiology and Biotechnology* (27); pp. 545-548.

Kötter, P., R. Amore, C.P. Hollenberg, and M. Ciriacy. (1990). "Isolation and Characterization of *Pichia stipitis* Xylitol Dehydrogenase Gene, XYL2, and Construction of a Xylose-Utilizing *Saccharomyces cerevisiae* Transformant." *Current Genetics,* 18:493-500.

Lachke, A.H.; Jeffries, T.W. (1986). "Levels of Enzymes of the Pentose Phosphate Pathway in *Pachysolen tannophilus* Y-2460 and Selected Mutants." *Enzyme and Microbial Technology* (8); pp. 353-359.

Lacis, L.S.; Lawford, H.G. (1989). "Analysis of the Variation of Ethanol Yield from Glucose or Xylose with Continuously Grown *Thermoanaerobacter Ethanolicus*." *Applied Biochemistry and Biotechnology* (20/21); pp. 479-490.

Lacis, L.S.; Lawford, H.G. (1988). "Ethanol Production from Xylose by *Thermoanaerobacter Ethanolicus* in Batch and Continuous Culture." *Archives of Microbiology* (150); pp. 48-55.

Lawford, H.G.; Rousseau, J.D. (1991a). "Ethanol Production by Recombinant *Escherichia coli* Carrying Genes from *Zymomonas mobilis*." *Applied Biochemistry and Biotechnology* (28/29); pp. 221-236.

Lawford, H.G.; Rousseau, J.D. (1991b). "Fuel Ethanol from Hardwood Hemicellulose Hydrolysate by Genetically Engineered *Escherichia coli* B Carrying Genes from *Zymomonas mobilis*." *Biotechnology Letters* (13); pp. 191-196.

Lawford, H.G.; Rousseau, J.D. (1991c). "Xylose to Ethanol: Enhanced Yield and Productivity using Genetically Engineered *Escherichia coli*." Paper presented at Energy from Biomass and Wastes XV, Washington, DC, March.

Ligthelm, M.E.; Prior, B.A.; du Preez, J.C.; Brandt, V. (1988a). "An Investigation of D-{1-^{13}C} Xylose Metabolism in *Pichia stipitis* under Aerobic and Anaerobic Conditions." *Applied Microbiology and Biotechnology* (28); pp. 293-296.

Ligthelm, M.E.; Prior, B.A.; du Preez, J.C. (1988b). "The Induction of **D**-xylose Catabolizing Enzymes in *Pachysolen tannophilus* and the Relationship to Anaerobic **D**-xylose Fermentation." *Biotechnology Letters* (10:3); pp. 207-212.

Ligthelm, M.E.; Prior, B.A.; du Preez, J.C. (1988c). "The Oxygen Requirements of Yeasts for the Fermentation of **D**-xylose and **D**-glucose to Ethanol." *Applied Microbiology and Biotechnology* (28); pp. 63-68.

Lighthelm, M.E.; Prior, B.A.; du Preez, J.C. (1988d). "The Effect of Respiratory Inhibitors on the Fermentative Ability of *Pichia stipitis, Pachysolen tannophilus* and *Saccharomyces cerevisiae* under Various Conditions of Aerobiosis." *Applied Microbiology and Biotechnology* (29); pp. 67-71.

Lohmeier-Vogel, E.; Hahn-Hägerdal, B. (1985). "The Utilization of Metabolic Inhibitors for Shifting Product Formation from Xylitol to Ethanol in Pentose Fermentations using *Candida tropicalis*." *Applied Microbiology and Biotechnology* (21); pp. 167-172.

Lucas, C.; van Uden, N. (1986). "Transport of Hemicellulose Monomers in the Xylose-fermenting Yeast *Candida shehatae*." *Applied Microbiology and Biotechnology* (23); pp. 491-495.

Lynd, L.R.; Ahn, H-J.; Anderson, G.; Hill, P.; Kersey, D.S.; Klapatch, T. (1991). "Thermophilic Ethanol Production-Investigation of Ethanol Yield and Tolerance in Continuous Culture." *Applied Biochemistry and Biotechnology* (28/29); pp. 549-570.

Magee, R.J.; Kosaric, N. (1985). " Bioconversion of Hemicellulosics." *Advances in Biochemical Engineering-Biotechnology* (32); pp. 61-93.

Maleszka, R.; Schneider, H. (1982). "Fermentation of **D**-xylose Xylitol and **D**-xylulose by Yeasts." *Canadian Journal of Microbiology* (28); pp. 360-363.

Manderson, G.J., ed. (1985). "CPD-5: the Conversion of C5 Sugars to Ethanol." EA/FE CPD-5 Report No. 1.

McCracken, L.D.; Gong, C-S. (1983). "**D**-xylose Metabolism by Mutant Strains of *Candida* sp." *Advances in Biochemical Engineering-Biotechnology* (27); pp. 33-55.

Morikawa, Y.; Takasawa, S.; Masunaga, I.; Takayama, K. (1985). "Ethanol Productions (sic) from **D**-xylose and Cellobiose by *Kluyveromyces cellobiovorus*." *Biotechnology and Bioengineering* (27); pp. 509-513.

Mountfort, D.O.; Rhodes, L.L. (1991). "Anaerobic Growth and Fermentation Characteristics of *Paecilomyces lilacinus* Isolated from Mullet Gut." *Applied and Environmental Microbiology* (57:7); pp. 1963-1968.

Neale, A.D.; Scopes, R.K.; Kelly, J.M. (1988). "Alcohol Production from Glucose and Xylose using *Escherichia coli* Containing *Zymomonas mobilis* Genes." *Applied Microbiology and Biotechnology* (29); pp. 162-167.

Nigam, J.N.; Ireland, R.S.; Margaritis, A.; Lachance, M.A. (1985). "Isolation and Screening of Yeasts that Ferment **D**-xylose Directly to Ethanol." *Applied and Environmental Microbiology* (50); pp. 1486-1489.

Ohta, K.; Beall, D.S.; Mejia, J.P.; Shanmugam, K.T.; Ingram, L.O. (1991). "Genetic Improvement of *Escherichia coli* for Ethanol Production: Chromosomal Integration of *Zymomonas mobilis* Genes Encoding Pyruvate Decarboxylase and Alcohol Dehydrogenase II." *Applied and Environmental Microbiology* (57:4); pp. 893-900.

Ohta, K.; Alterthum, F.; Ingram, L.O. (1990). "Effects of Environmental Conditions on Xylose Fermentation by Recombinant *Escherichia coli.*" *Applied and Environmental Microbiology* (56); pp. 463-465.

Olsson, L.; Lindén, T.; Hahn-Hägerdal, B. (1992). "Performance of Microorganisms in Spent Sulphite Liquor and Enzymatic Hydrolysate of Steam Pretreated *Salix.*" *Applied Biochemistry and Biotechnology* (34/35); pp. 359-368.

Parekh, S.R.; Yu, S.; Wayman, M. (1986). "Adaptation of *Candida shehatae* and *Pichia stipitis* to Wood Hydrolyzates for Increased Ethanol Production." *Applied Microbiology and Biotechnology* (25); pp. 300-304.

Prior, B.A.; Kilian, S.G.; duPreez, J.C. (1989). "Fermentation of **D**-xylose by the Yeasts *Candida shehatae* and *Pichia stipitis.*" *Process Biochemistry* (Feb); pp. 21-32

Posno, M.; Heuvelmans, P.T.H.M.; van Giezen, M.J.F.; Lockman, B.C.; Leer, R.J.; Pouwels, P.H. (1991). "Complementation of the Inability of *Lactobacillus* Strains to Utilize **D**-xylose with **D**-xylose Catabolism-Encoding Genes of *Lactobacillus pentosus." Applied and Environmental Microbiology* (57); pp. 2764-2766.

Rosenberg, S.L.; Batter, T.R.; Blanch, H.W.; Wilke, C.R. (1981). "Hemicellulose Utilization for Ethanol Production." *AICHE Symposium Series No. 207* (77); pp. 107-114.

Saddler, J.N.; Yu, E.K.C.; Mes-Hartree, M.; Levitin, N.; Brownell, H.H. (1983). "Utilization of Enzymatically Hydrolyzed Wood Hemicelluloses by Microorganisms for Production of Liquid Fuels." *Applied and Environmental Microbiology* (45); pp. 153-160.

Sarthy, A.V.; McConaughy, B.L.; Lobo, Z.; Sundstrum, J.A.; Furlong, C.E.; Hall, B.D. (1987). "Expression of the *Escherichia coli* Xylose Isomerase Gene in *Saccharomyces cerevisiae.*" *Applied and Environmental Microbiology* (53:9); pp. 1996-2000.

Schneider, H. (1989). "Conversion of Pentoses to Ethanol by Yeasts and Fungi." *Critical Reviews in Biotechnology* (9:1); pp. 1-2.

Sims, A.P.; Barnett, J.A. (1978). "The Requirement of Oxygen for the Utilization of Maltose Cellobiose and **D**-galactose by Certain Anaerobically Fermenting Yeasts (Kluyver effect)." *Journal of General Microbiology* (106); pp. 277-288.

Singh, A.; Kumar, P.K.R. (1991). "*Fusarium oxysporum:* Status in Bioethanol Production." *Critical Reviews in Biotechnology* (11:2); pp. 129-147.

Singh, A.; Kumar, P.K.R.; Schügerl, K. (1991). "Shift in Product Formation from Acetate to Ethanol using Metabolic Inhibitors in *Fusarium oxysporum*." *Biotechnology Letters* (13:7); pp. 527-532.

Skoog, K.; Jeppsson, H.; Hahn-Hägerdal, B. (1991). "The Effect of Oxygenation on Glucose Fermentation with *Pichia stipitis*." *Applied Biochemistry and Biotechnology* (34/35); pp. 369-375.

Skoog, K.; Hahn-Hägerdal, B. (1990). "Effect of Oxygenation on Xylose Fermentation by *Pichia stipitis*." *Applied and Environmental Microbiology* (56); pp. 3389-3394.

Skoog, K.; Hahn-Hägerdal, B. (1988). "Xylose Fermentation." *Enzyme and Microbial Technology* (10); pp. 66-80.

Slininger, P.J.; Bothast, R.J.; Okos, M.R.; Ladisch, M.R. (1985). "Comparative Evaluation of Ethanol Production by Xylose-Fermenting Yeasts Presented High Xylose Concentrations." *Biotechnology Letters* (7:6); pp. 431-436.

Sreenath, H.K.; Jeffries, T.W. (1987). "Batch and Membrane-assisted Cell Recycling in Ethanol Production by *Candida shehatae*." *Biotechnology Letters* (9); pp. 293-298.

Strasser, A.W.M.; Dahlems, U.M.; Kötter, P.; Ciriacy, M.; Metzger, M.; Hollenberg, C.P. (1991). "Genetically Engineered *Saccharomyces cerevisiae* for Improvement of Bioethanol Production Processes." Paper presented at the 13th Symposium on Biotechnology for Fuels and Chemicals.

Stryer, L. (1981). *Biochemistry: Second Ed.* San Francisco, CA: Freeman & Co.

Suihko, M.L.; Enari, T.M. (1981). "The Production of Ethanol from **D**-glucose and **D**-xylose by Different *Fusarium* Strains." *Biotechnology Letters* (3); pp. 723-728.

Suihko, M.L.; Dražić, M. (1983). "Pentose Fermentation by Yeasts." *Biotechnology Letters* (5); pp. 107-112.

Takuma, S.; Nakashima, N.; Tantirungkij, M.; Kinoshita, S.; Okada, H.; Seki, T.; Yoshida, T. (1991). "Isolation of Xylose Reductase Gene of *Pichia stipitis* and its Expression in *Saccharomyces cerevisiae*." *Applied Biochemistry and Biotechnology* (28/29); pp. 327-340.

Toivola, A.; Yarrow, D.; Van den Bosch, E.; Van Dijken, J.P.; Scheffers, W.A. (1984). "Alcoholic Fermentation of **D**-xylose by Yeasts." *Applied and Environmental Microbiology* (47); pp. 1221-1223.

Tolan, J.S.; Finn, R.K. (1987a). "Fermentation of **D**-xylose and L-arabinose to Ethanol by *Erwinia chrysanthemi.*" *Applied and Environmental Microbiology* (53:9); pp. 2033-2038.

Tolan, J.S.; Finn, R.K. (1987b). "Fermentation of **D**-xylose to Ethanol by Genetically Modified *Klebsiella planticola*." *Applied and Environmental Microbiology* (53:9); pp. 2039-2044.

Verduyn, C.; Frank, F. Jzn.; van Dijken, J.P.; Scheffers, W.A. (1985a). "Multiple Forms of Xylose Reductase in *Pachysolen tannophilus* CBS4044." *FEMS Microbiology Letters* (30); pp. 313-317.

Verduyn, C.; van Kleef, R.; Frank, J.; Schreuder, H.; van Dijken, J.P.; Scheffers, W.A. (1985b). "Properties of the NAD(P)H-dependent Xylose Reductase from the Xylose-Fermenting Yeast *Pichia stipitis*." *The Biochemical Journal* (226); pp. 669-677.

Webb, S.R.; Lee, H. (1990). "Regulation of **D**-xylose Utilization by Hexoses in Pentose-fermenting Yeasts." *Biotechnology Advances* (8); pp. 685-697.

Wu, J.F.; Lastick, S.M.; Updegraff, D.M. (1986). "Ethanol Production from Sugars Derived from Plant Biomass by a Novel Fungus." *Nature* (321); pp. 887-888.

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The U.S. Department of Energy/National Renewable Energy Laboratory Ethanol from Biomass Program is evaluating a variety of fermentation-based bioconversion processes for converting xylose and other pentose sugars to ethanol at an industrial scale. The past several years have seen tremendous progress in the understanding of xylose metabolism and in the identification, characterization, and development of strains with improved xylose fermentation characteristics. This report reviews the current state of xylose fermentation technology, focusing on recent improvements in the direct fermentation of xylose.

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